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ENVIRONMENTAL MICROBIOLOGY

TroR Negatively Regulates the TroABCD System and Is Required for Resistance to Metal Toxicity and Virulence in *Streptococcus suis*

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ABSTRACT *Streptococcus suis* is an emerging zoonotic pathogen that causes severe swine and human infections. Metals are essential nutrients for life; however, excess metals are toxic to bacteria. Therefore, maintenance of intracellular metal homeostasis is important for bacterial survival. Here, we characterize a DtxR family metalloregulator, TroR, in *S. suis*. TroR is located upstream of the *troABCD* operon, whose expression was found to be significantly downregulated in response to excess manganese (Mn). Deletion of *troR* resulted in reduced growth when *S. suis* was cultured in metal-replete medium supplemented with elevated concentrations of zinc (Zn), copper (Cu), or cobalt (Co). Mn supplementation could alleviate the growth defects of the Δ*troR* mutant under Zn and Co excess conditions; however, it impaired the growth of the wild-type (WT) and complemented (CΔ*troR*) strains under Cu excess conditions. The growth of Δ*troR* was also inhibited in metal-depleted medium supplemented with elevated concentrations of Mn. Moreover, the Δ*troR* mutant accumulated increased levels of intracellular Mn and Co, rather than Zn and Cu. Deletion of *troR* in *S. suis* led to significant upregulation of the *troABCD* operon. Furthermore, *troA* expression in the WT strain was induced by ferrous iron [Fe(II)] and Co and repressed by Mn and Cu; the repression of *troA* was mediated by TroR. Finally, TroR is required for *S. suis* virulence in an intranasal mouse model. Together, these data suggest that TroR is a negative regulator of the TroABCD system and contributes to resistance to metal toxicity and virulence in *S. suis*.

IMPORTANCE Metals are essential nutrients for life; however, the accumulation of excess metals in cells can be toxic to bacteria. In the present study, we identified a metalloregulator, TroR, in *Streptococcus suis*, which is an emerging zoonotic pathogen. In contrast to the observations in other species that TroR homologs usually contribute to the maintenance of homeostasis of one or two metals, we demonstrated that TroR is required for resistance to the toxicity conferred by multiple metals in *S. suis*. We also found that deletion of *troR* resulted in significant upregulation of the *troABCD* operon, which has been demonstrated to be involved in manganese acquisition in *S. suis*. Moreover, we demonstrated that TroR is required for the virulence of *S. suis* in an intranasal mouse model. Collectively, these results suggest that TroR is a negative regulator of the TroABCD system and contributes to resistance to metal toxicity and virulence in *S. suis*.

KEYWORDS *Streptococcus suis*, TroR, TroABCD, repression, metal toxicity, virulence

Metals, such as iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu), are essential nutrients for almost all organisms because of their role as enzymatic cofactors or protein structural components (1, 2). Humans and other mammals can sequester and/or

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mobilize essential metals to make them unavailable to invading pathogens (3). This process, termed nutritional immunity, is an important strategy used by the host to respond to bacterial infection (3, 4). To overcome this and infect the host, bacteria have developed high-affinity metal transporters for acquiring metals from diverse sources (5, 6). Although the effective uptake of metals is critical for bacterial survival within the host, the overaccumulation of metals is detrimental to bacterial cells (6). Emerging evidence indicates that hosts also utilize metal toxicity as an immune defense mechanism against bacterial pathogens (7, 8). As a countermeasure, bacteria have evolved complex mechanisms, such as the efflux of excess metals, to maintain metal homeostasis (8). As such, there is increasing evidence that metal homeostasis is essential for the physiology and/or pathogenesis of bacterial pathogens (9–16).

Streptococcus suis is an emerging zoonotic bacterial pathogen that is associated with severe swine and human diseases, such as meningitis, septicemia, and endocarditis (17, 18). Among the 29 serotypes of *S. suis* (1 to 19, 21, 23 to 25, 27 to 31, and 1/2) described according to the capsular polysaccharides, *S. suis* serotype 2 (*S. suis* 2) is the most prevalent serotype and is the most common cause of swine and human infections worldwide (19). *S. suis* was the most prevalent bacterial pathogen to be isolated from pig farms in China from 2013 to 2017 (isolation rate, 16.9%) and the third most common bacterial pathogen to be isolated from porcine laboratory submissions in New Zealand from June 2003 to February 2016 (isolation rate, 5.5%) (20, 21). Moreover, in one study, it could be isolated from 95% of the sampled Swedish grower pigs (22). While *S. suis* is responsible for huge economic losses in the swine industry worldwide, it poses serious threats to public health. Since the report of a human case of *S. suis* infection in Denmark in 1968, human cases have reached over 1,600 worldwide by the end of 2013; some of these were fatal (23). Sporadic cases of *S. suis* infections in humans have been frequently reported worldwide in recent years as well (24–29). Thus, a better understanding of the physiology and pathogenesis of *S. suis* is urgent for the control of the infection caused by this bacterium.

It has been established that metal acquisition and the maintenance of metal homeostasis are important for the physiology and pathogenesis of *S. suis*. Dpr-mediated removal of intracellular ferrous iron [Fe(II)] protects *S. suis* against H_2O_2-induced oxidative stress (30). The Fe transporter FeoB and two cation-uptake regulators (AdcR and Fur) contribute to the virulence of *S. suis* (31, 32). Both the Mn acquisition system TroA and efflux system MntE are involved in counteracting oxidative stress and virulence in *S. suis* (33, 34). Recently, we showed that PmtA, an Fe(II) and cobalt (Co) efflux system in *S. suis*, plays a role in resistance to H_2O_2-induced oxidative stress (16). We also demonstrated that CopA, a Cu-transporting ATPase, protects *S. suis* against Cu-induced bactericidal effect (35). In addition, Zur, a Zn uptake regulator, is involved in *S. suis* response to Zn toxicity (36). Despite some progress, the mechanisms underlying metal acquisition and the maintenance of metal homeostasis in *S. suis* remain poorly understood. For example, little is known about the regulators by which *S. suis* senses and responds to excess metals other than Zn.

In attempts to explore the transcriptome change in *S. suis* in response to excess Mn and to elucidate the mechanisms underlying the maintenance of Mn homeostasis, we identified a DtxR family metalloregulator, TroR, in *S. suis*. Unlike the homologs in other species that are generally specific to Mn, *S. suis* TroR contributes to resistance to the toxicity conferred by Mn, Zn, Cu, and Co. We also investigated the regulon of TroR in *S. suis* and found that TroR negatively regulates the *troABCD* operon. More importantly, TroR is required for the virulence of *S. suis* in an intranasal mouse model.

RESULTS

***S. suis* downregulates the expression of the *troABCD* operon in response to excess Mn.** To explore the mechanisms underlying the maintenance of Mn homeostasis, we performed transcriptome analysis of *S. suis* cultured in the presence of 1 mM $MnSO_4$ in comparison with that cultured in the presence of water by RNA sequencing. Most *S. suis* genes exhibited no significant difference in expression levels following treatment with Mn. Of the 25 genes that were significantly differentially expressed (fold change of >2, adjusted *P* value [*P*adj] of <0.05), 16 genes were upregulated, and

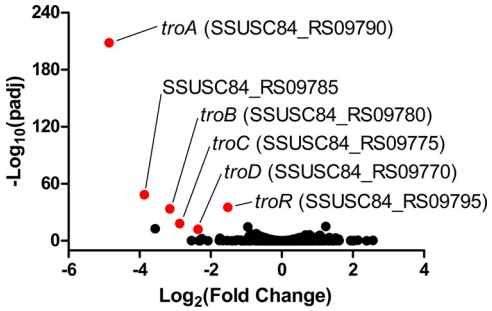

FIG 1 The *S. suis* transcriptome is changed in response to excess Mn. The *S. suis* strain SC19 was grown to the mid-exponential phase and exposed to 1 mM $MnSO_4$ or deionized water. After 15 min of treatment, RNA was isolated and subjected to RNA sequencing analysis. The figure shows the transcription profile of *S. suis* in the presence of Mn in comparison with that in the presence of water. The genes with a fold change of >2 and an adjusted *P* value (*P*adj) of <0.05 were defined as differentially expressed genes.

the remaining nine genes were downregulated (see Table S1 in the supplemental material). There was a 5.12- to 29.05-fold decrease in the expression levels of genes encoding a well-characterized ABC transporter, TroABCD (Fig. 1 and Table S1). Moreover, there was a 2.87-fold decrease in the expression level of *troR*, a gene predicted to encode a transcriptional repressor of the *troABCD* operon, after treatment with Mn (Fig. 1 and Table S1). TroA has the ability to bind Mn and Zn and is required for Mn acquisition in *S. suis* (33, 37). We hypothesized that the repression of the TroABCD system by TroR is an important mechanism by which *S. suis* maintains Mn and Zn homeostasis.

***In silico* analysis of TroRABCD-like systems in *S. suis* and other species.** TroR is one of the DtxR family metalloregulators; members of this family have been extensively characterized in streptococci and several other prokaryotes (38). Phylogenetic comparison of *S. suis* TroR with the previously identified DtxR family metalloregulators revealed that *S. suis* TroR is very closely related to its homologs in streptococci, including *Streptococcus pyogenes* MtsR (39–41), *Streptococcus pneumoniae* PsaR (42–45), *Streptococcus gordonii* ScaR (46, 47), *Streptococcus parasanguinis* FimR (48), and *Streptococcus mutans* SloR (49–51) (see Fig. S1 in the supplemental material). Protein sequence alignment analysis revealed that *S. suis* TroR shares approximately 54 to 60% amino acid identity to its homologs in these streptococci.

The genetic structures of TroRABCD-like systems vary across streptococci (Fig. 2). In

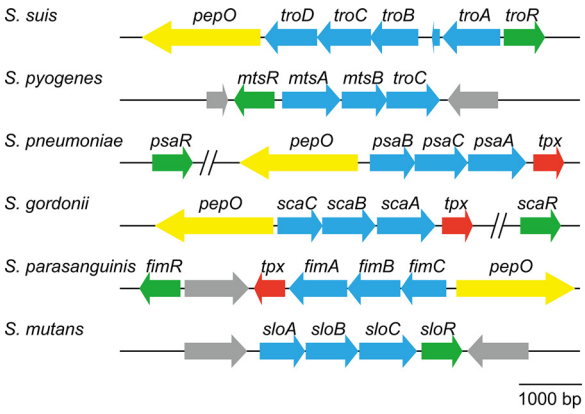

FIG 2 Genetic structures of TroRABCD-like systems in streptococci. Arrows indicate the direction of transcription, and break lines represent great distance in the genome. Genetic structure analyses were according to the following genomes: *S. suis* SC84, NC_012924.1; *S. pyogenes* M1, NC_002737.2; *S. pneumoniae* D39, NC_008533.2; *S. gordonii* CH1, NC_009785.1; *S. parasanguinis* FW213, NC_017905.1; and *S. mutans* UA159, NC_004350.2.

S. suis and *S. pyogenes*, the *troR* homologs and *troABCD*-like operons are adjacent, but transcribed in opposite directions (Fig. 2). In *S. pneumoniae* and *S. gordonii*, the *troR* homologs are far away from the *troABCD*-like operons (Fig. 2). Unlike the genetic organization in these species, the *troR* homologs and *troABCD*-like operons are adjacent and transcribed in the same direction in *S. parasanguinis* and *S. mutans* (Fig. 2). In *S. pneumoniae*, *S. gordonii*, and *S. parasanguinis*, *pepO* (encoding metalloendopeptidase) and *tpx* (encoding thiol peroxidase) are located upstream and downstream of the *troABCD*-like operons, respectively (Fig. 2). While only *pepO* is located downstream of the *troABCD* operon in *S. suis*, neither of the two genes is located upstream or downstream of the *troABCD*-like operons in *S. pyogenes* and *S. mutans* (Fig. 2).

TroR is required for *S. suis* resistance to Zn, Co, and Cu toxicity during growth in metal-replete medium. To investigate the role of TroR, we created a *troR* deletion mutant (Δ*troR*) (see Fig. S2A in the supplemental material) from the *S. suis* 2 strain SC19 (52). A functional complementation strain of the mutant (CΔ*troR*), in which *troR* was placed under its native promoter, was also generated. The two strains were verified by PCR (Fig. S2B), reverse transcription-PCR (Fig. S2C), and DNA sequencing (data not shown).

TroA, a lipoprotein potentially repressed by TroR, can interact with Mn and Zn and is involved in Mn acquisition in *S. suis* (33, 37). The homologs of TroR control Mn homeostasis in some prokaryotes, such as *S. pyogenes* (39), *Staphylococcus aureus* (11), *Mycobacterium tuberculosis* (53), and *Treponema denticola* (54). Considering these observations, we first assessed the role of TroR in Mn and Zn homeostasis in *S. suis*. The wild-type (WT), Δ*troR* mutant, and CΔ*troR* complemented strains were grown in TSBS (tryptic soy broth supplemented with newborn bovine serum) medium (metal replete) supplemented with or without elevated concentrations of Mn/Zn, and their growth was evaluated. In the absence of metal supplementation, the three strains exhibited similar growth curves (Fig. 3A). Compared with the WT and CΔ*troR* strains, the Δ*troR* strain exhibited only a moderate growth defect in the presence of a high concentration of Mn (4 mM) (Fig. 3B). However, the Δ*troR* strain showed a growth defect in the presence of as little as 50 μM Zn; the level of inhibition was more severe in the presence of higher concentrations of Zn (Fig. 3C).

To assess whether TroR is also involved in resistance to the toxicity conferred by other metals, the growth curves of the three strains were also assessed in the presence of elevated concentrations of Fe(II), Cu, Co, nickel (Ni), calcium (Ca), or magnesium (Mg). The Δ*troR* strain displayed obvious growth inhibition when supplemented with Cu or Co (Fig. 3D and E). However, it showed only a moderate growth defect in the presence of high concentrations of Fe(II), Ni, Ca, or Mg (4 mM) (see Fig. S3 in the supplemental material).

Taken together, the Δ*troR* mutant exhibited obviously impaired growth in TSBS medium supplemented with Zn, Co, and Cu, indicating that TroR protects *S. suis* against Zn, Co, and Cu toxicity under metal-replete conditions.

Mn supplementation improves the growth of Δ*troR* under Zn or Co excess conditions. In a previous study, we found that the growth defect of a Δ*pmtA* mutant under Fe(II) or Co excess conditions could be alleviated by the addition of Mn (16). Accordingly, we sought to determine whether Mn supplementation could alleviate the growth defect of the Δ*troR* mutant under Zn, Cu, and Co conditions. While the growth of the Δ*troR* mutant was markedly inhibited by 100 μM Zn, Mn supplementation improved the growth of the Δ*troR* strain under Zn excess conditions (Fig. 4A to C). Similarly, Mn supplementation alleviated the growth defect of the Δ*troR* strain under Co excess conditions; however, the effect was not as significant as that under Zn excess conditions (Fig. 4D to F). Unlike the observations under Zn and Co excess conditions, Mn supplementation had no rescue effect on the growth defect of the Δ*troR* strain under Cu excess conditions. Moreover, Mn addition resulted in impaired growth of the WT and CΔ*troR* complemented strains (Fig. 4G to I).

TroR contributes to *S. suis* growth under Mn excess conditions when cultured in metal-depleted medium. Because TroR homologs contribute to Mn homeostasis in other prokaryotes (11, 39, 53, 54), and the toxicity of a metal might be rescued by

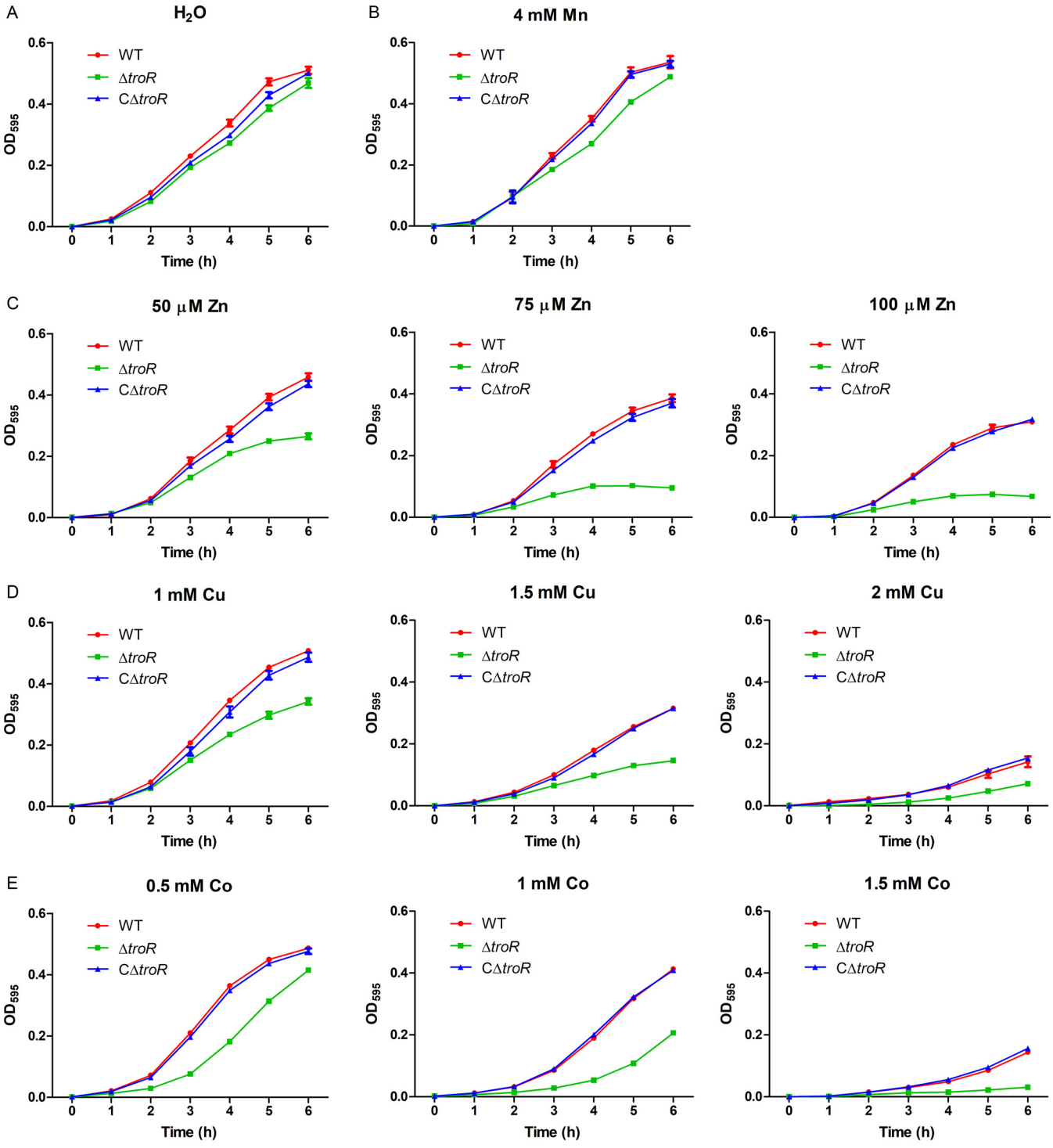

FIG 3 TroR is required for *S. suis* growth in metal-replete medium supplemented with excess Zn, Cu, or Co. The WT, Δ*troR*, and CΔ*troR* strains were grown in TSBS medium supplemented with deionized water (A), 4 mM Mn (B), or various concentrations of Zn (C), Cu (D), or Co (E). The strains were grown in 96-well plates (200 μl per well) at 37°C with linear shaking, and the OD_{595} values were measured hourly. At least three independent experiments were performed; the data shown are the means ± standard deviations (SDs) from three wells in a representative experiment.

another metal, we hypothesized that in metal-replete medium, the effect of Mn supplementation on the growth of the Δ*troR* mutant might be masked by other metals existing in this medium. To test this hypothesis, we monitored the growth of the WT, Δ*troR*, and CΔ*troR* complemented strains in Chelex-treated TSBS medium (metal depleted) supplemented with elevated concentrations of Mn. In the absence of Mn,

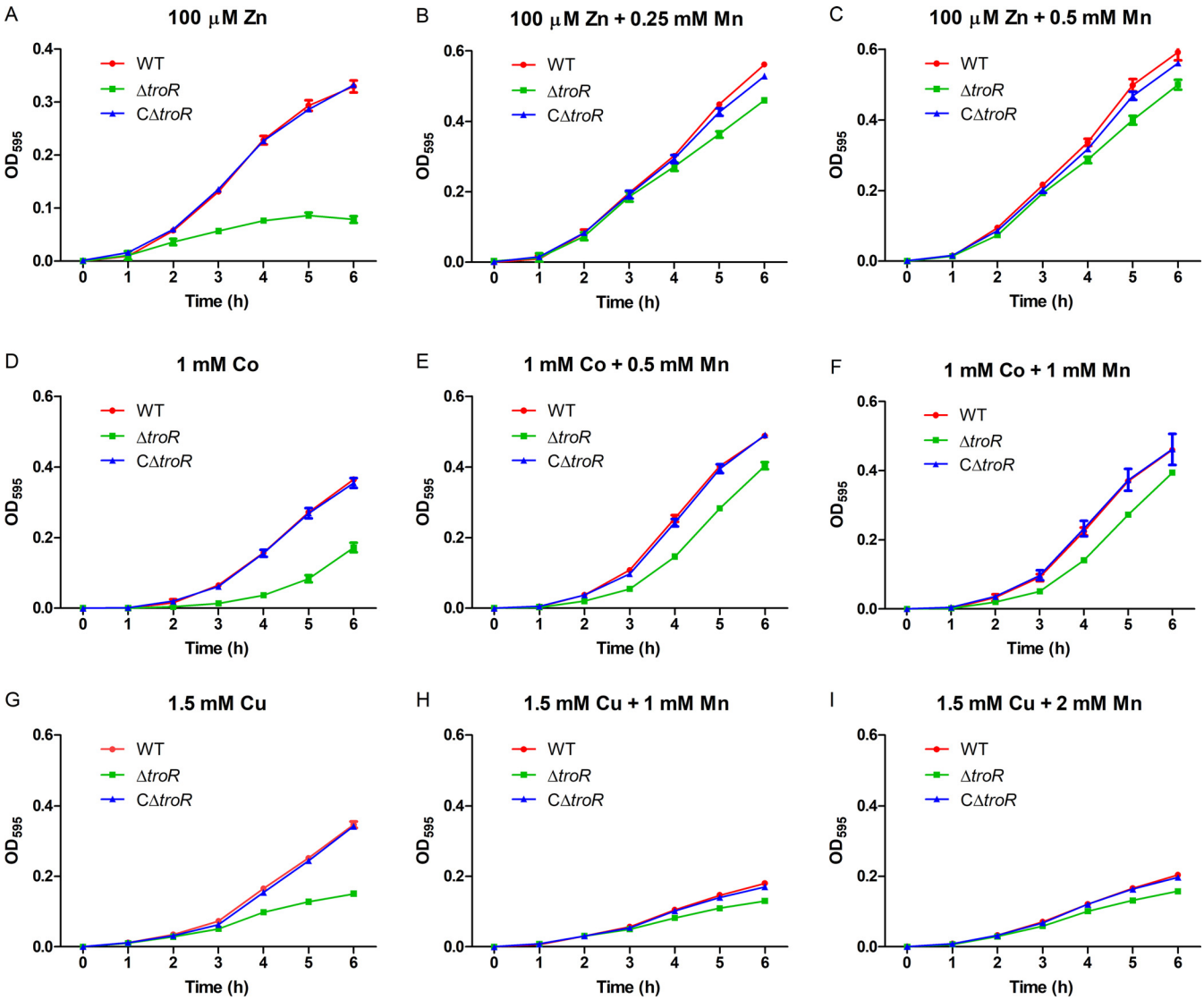

FIG 4 Mn supplementation rescues the growth defect of the Δ*troR* mutant under Zn or Co excess conditions. (A to C) The WT, Δ*troR*, and CΔ*troR* strains were grown in TSBS medium supplemented with 100 μM Zn alone (A), 100 μM Zn with 0.25 mM Mn (B), or 0.5 mM Mn (C). (D to F) The WT, Δ*troR*, and CΔ*troR* strains were grown in TSBS medium supplemented with 1 mM Co alone (D), 1 mM Co with 0.5 mM Mn (E), or 1 mM Mn (F). (G to I) The WT, Δ*troR*, and CΔ*troR* strains were grown in TSBS medium supplemented with 1.5 mM Cu alone (G), 1.5 mM Cu with 1 mM Mn (H), or 2 mM Mn (I). The strains were grown in 96-well plates (200 μl per well) at 37°C with linear shaking, and the OD_{595} values were measured hourly. At least three independent experiments were performed; the data shown are the means ± SDs from three wells in a representative experiment.

the growth rates of the three strains were almost comparable, with a very slight growth defect being observed in the Δ*troR* strain (Fig. 5A). However, when supplemented with various concentrations of Mn, the Δ*troR* strain displayed remarkable growth inhibition in comparison with the WT and CΔ*troR* strains (Fig. 5B to D). These results indicate that TroR is required for *S. suis* resistance to Mn toxicity during growth under metal-depleted conditions.

TroR deletion results in increased levels of intracellular Mn and Co, rather than Zn and Cu. To better understand the mechanism underlying the role of TroR in resistance to metal toxicity, we analyzed the levels of intracellular metals in the WT, Δ*troR* mutant, and CΔ*troR* complemented strains by inductively coupled plasma-optical emission spectroscopy (ICP-OES). We grew the *S. suis* strains in metal-replete medium to an optical density at 600 nm (OD_{600}) of 0.6, supplemented the medium with Mn, Zn, Cu, or Co, cultured the cells for another 30 min, and collected the cells for ICP-OES analysis. When cultured in the presence of Mn, the Δ*troR* mutant accumulated significantly higher levels of

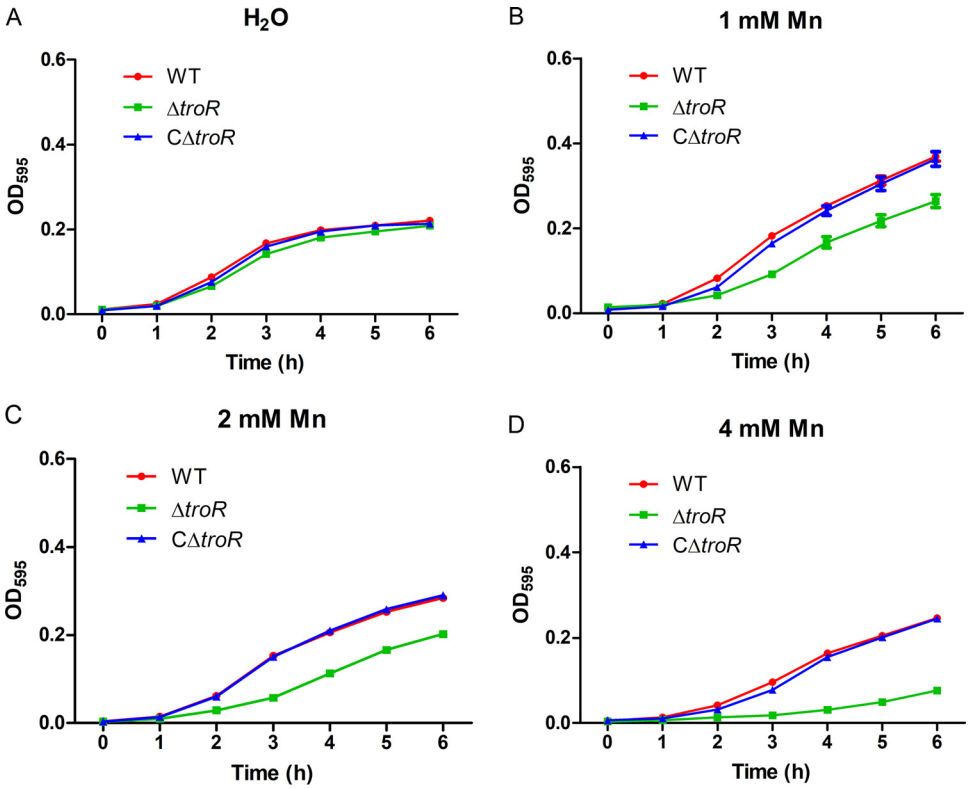

FIG 5 TroR is required for *S. suis* growth in metal-depleted medium supplemented with excess Mn. The WT, Δ*troR*, and CΔ*troR* strains were grown in Chelex-treated TSBS medium supplemented with deionized water (A), 1 mM Mn (B), 2 mM Mn (C), or 4 mM Mn (D). The strains were grown in 96-well plates (200 μl per well) at 37°C with linear shaking, and the OD_{595} values were measured hourly. At least three independent experiments were performed; the data shown are the means ± SDs from three wells in a representative experiment.

intracellular Mn than the WT and CΔ*troR* complemented strains (Fig. 6A). Similarly, the level of intracellular Co was significantly higher in the Δ*troR* strain than in the WT and CΔ*troR* strains when cultured in the presence of Co (Fig. 6D). Nevertheless, the three strains accumulated comparable levels of intracellular Zn and Cu when cultured in the presence of Zn and Cu, respectively (Fig. 6B and C). Hence, the high susceptibility of the Δ*troR* strain to Mn and Co toxicity might be due to the overload of intracellular Mn and Co, respectively.

RNA sequencing analysis of the genes regulated by TroR in *S. suis*. To identify the regulon of TroR in *S. suis* and to elucidate the mechanisms underlying TroR-mediated resistance to metal toxicity, we assessed the global gene transcription profiles of the WT and Δ*troR* mutant strains grown in TSBS medium by RNA sequencing. In total, 20 genes were significantly differentially expressed in the Δ*troR* strain, with 16 genes being upregulated and the remaining four genes being downregulated (Table 1). The gene encoding a serine protease was the most upregulated gene in the Δ*troR* strain (183.03-fold) (Table 1). As expected, the genes in the *troABCD* operon were upregulated by approximately 10.20- to 30.03-fold in the Δ*troR* strain (Table 1). The gene encoding CopA, a Cu efflux system (35), was upregulated by approximately 2.29-fold in the mutant (Table 1). Moreover, *pmtA*, which encodes an Fe(II) and Co efflux pump in *S. suis* (16), was also upregulated in the Δ*troR* strain (3.67-fold) (Table 1). We found that three of the downregulated genes (i.e., GeneID no. SSUSC84_RS08465, SSUSC84_RS08475, and SSUSC84_RS08495) encode enzymes involved in fatty acid biosynthesis (Table 1).

Expression of *troA* in the WT and Δ*troR* strains in response to various metals. To determine which metals are correlated with *troA* expression and to further assess the role of TroR in the repression of the *troABCD* operon, the expression levels of *troA* in the WT and Δ*troR* strains treated with various metals were determined by reverse

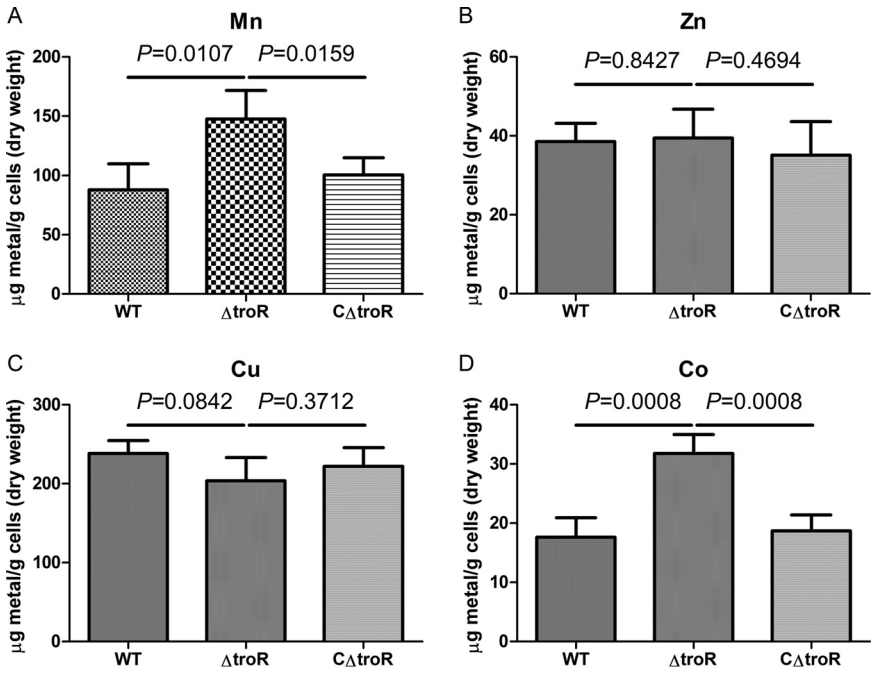

FIG 6 The Δ*troR* strain accumulates significantly increased levels of intracellular Mn and Co, rather than Zn and Cu. The WT, Δ*troR*, and CΔ*troR* strains were grown in TSBS medium to an OD_{600} of 0.6 and supplemented with 4 mM Mn (A), 100 μM Zn (B), 1.5 mM Cu (C), or 1 mM Co (D). The cultures were incubated for another 30 min, and bacterial cells were then collected to measure the metal contents. The metal content was expressed as μg of metal per g of cells (dry weight). The data shown are the means ± SDs from four biological samples.

transcription-quantitative PCR analysis. As reported previously (33, 55), *troA* expression in the WT strain was significantly induced by Fe(II) and repressed by Mn (Fig. 7). Although TroA has the ability to bind Zn (37), no significant difference in levels of *troA* expression was detected when the WT strain was treated with Zn (Fig. 7). Similar to the case of Fe(II), Co treatment resulted in approximately 3-fold upregulation of *troA* in the WT strain (Fig. 7). In contrast, the expression level of *troA* in the WT strain was

TABLE 1 Summary of the differentially expressed genes in Δ*troR* compared to the WT strain

	Read count for:				
GeneID	Δ*troR* mutant	WT	Log_2 fold change	*P*adj	Description
SSUSC84_RS08475	172.496259	1,692.640537	−3.2946	4.98E−05	Enoyl-[acyl-carrier-protein] reductase FabK
SSUSC84_RS08465	79.42321497	776.5504876	−3.2894	0.032699	3-Oxoacyl-[acyl-carrier-protein] reductase
SSUSC84_RS08495	874.0360026	2,634.921548	−1.592	1.92E−07	Enoyl-CoA hydratase
SSUSC84_RS07585	3,695.542083	6,997.785595	−0.92111	0.032699	Hypothetical protein
SSUSC84_RS01215	215.337514	106.7483999	1.0124	0.047586	α,α-Phosphotrehalase
SSUSC84_RS05475	181.1103214	85.95622599	1.0752	0.038677	β-Hexosamidase
SSUSC84_RS06535	1,163.244077	508.8348814	1.1929	0.00072886	CopA
SSUSC84_RS05645	319.8083029	135.5352753	1.2385	0.0019577	Membrane protein
SSUSC84_RS05470	235.5409301	99.05513486	1.2497	0.0037403	Glycoside hydrolase family 3 protein
SSUSC84_RS07610	1,581.638773	626.7822994	1.3354	9.38E−05	GtrA family protein
SSUSC84_RS04025	197.6334627	72.90937538	1.4387	0.00033251	4-Oxalocrotonate tautomerase
SSUSC84_RS01570	307.5378467	83.89864933	1.874	0.016174	PmtA
SSUSC84_RS09765	88,487.52168	8,676.327877	3.3503	6.53E−32	PepO
SSUSC84_RS09790	33,734.07384	2,622.592268	3.6851	4.54E−37	TroA
SSUSC84_RS09785	8,337.89467	433.2156108	4.2665	1.34E−46	Hypothetical protein
SSUSC84_RS10665	177.4460472	7.880123374	4.493	4.36E−25	Hypothetical protein
SSUSC84_RS09780	30,303.83419	1,307.754198	4.5343	3.60E−52	TroB
SSUSC84_RS09775	15,491.15677	554.916931	4.803	9.41E−57	TroC
SSUSC84_RS09770	16,903.09614	562.798094	4.9085	9.68E−59	TroD
SSUSC84_RS09305	46,886.28412	256.1670182	7.5159	9.56E−08	Serine protease

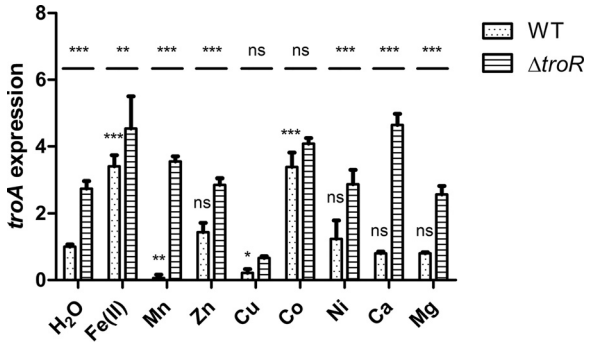

FIG 7 *troA* expression in the WT and Δ*troR* strains in response to various metals. The WT and Δ*troR* strains were grown in TSBS to an OD_{600} of 0.6 and treated for 15 min with 2 mM Fe(II), 2 mM Mn, 50 μM Zn, 1 mM Cu, 1 mM Co, 1 mM Ni, 2 mM Ca, 2 mM Mg, or deionized water. RNA was isolated to perform reverse transcription-quantitative PCR analysis. The data shown are the means ± SDs from three biological triplicates. *troA* expression in the WT strain in response to the metals was analyzed by one-way analysis of variance with Bonferroni's posttest. Two-way analysis of variance with Bonferroni's posttest was used for comparison of *troA* expression in the Δ*troR* mutant with that in the WT strain. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significant difference.

significantly downregulated by Cu treatment (Fig. 7). Nevertheless, Ni, Ca, and Mg treatments had no significant effect on the expression level of *troA* in the WT strain (Fig. 7). TroR functions as a repressor of the *troABCD* operon; thus, the deletion of *troR* resulted in the derepression of the *troABCD* operon. Correspondingly, the expression level of *troA* in the Δ*troR* mutant was commonly higher than that in the WT strain following treatment with or without the metals, with the exception of Cu and Co treatments (Fig. 7). These results indicate that *troA* expression is induced by Fe(II) and Co and repressed by Mn and Cu and that the repression is mediated by TroR.

***troR* deletion attenuates *S. suis* virulence in an intranasal mouse model.** TroR homologs have been shown to be critical for the virulence of certain bacterial species (39, 44, 56–58). Therefore, we assessed the role of TroR in the virulence of *S. suis* using two murine infection models. In a mouse model of intraperitoneal infection, four groups of BALB/c mice (10 mice per group) were infected by intraperitoneal injection of 3×10^8 CFU of the WT, Δ*troR* mutant, or CΔ*troR* complemented strain or with phosphate-buffered saline (PBS). After the injections, no clinical symptom was observed in the mice in the PBS-treated group. In contrast, the mice infected with *S. suis* in the remaining three groups exhibited typical clinical symptoms within 12 h after infection. The levels of the signs of infection were almost comparable among the three groups. Finally, 50% of the mice in the WT group and 40% in the Δ*troR* and CΔ*troR* groups survived over the course of the experiment (see Fig. S4 in the supplemental material). There were no significant differences in the survival rates between the Δ*troR* and WT groups ($P = 0.7984$) and between the Δ*troR* and CΔ*troR* groups ($P = 0.7246$). Thus, TroR plays no significant role in *S. suis* virulence in the mouse intraperitoneal infection model.

In the competitive-infection assay using a mouse intranasal infection model, groups of five BALB/c mice were intranasally infected with a 1:1 mixture of either the Δ*troR* and WT strains or the Δ*troR* and CΔ*troR* strains. The blood, heart, liver, spleen, lung, kidney, and brain samples from the infected mice were collected at 12 h after infection. For each sample collected from mice infected with the mixture of the Δ*troR* and WT strains, a total of 70 to 80 colonies were analyzed by colony PCR to determine the competitive index (CI: the ratio of Δ*troR* mutant to WT in each sample divided by that in the inoculum). For each sample collected from mice infected with the mixture of the Δ*troR* and CΔ*troR* strains, a total of 70 to 80 colonies were analyzed for spectinomycin susceptibility to determine the CI (the ratio of the Δ*troR* mutant to CΔ*troR* complemented strain in each sample divided by that in the inoculum). The results showed that the CI values were significantly less than 1 for all the samples examined (Fig. 8),

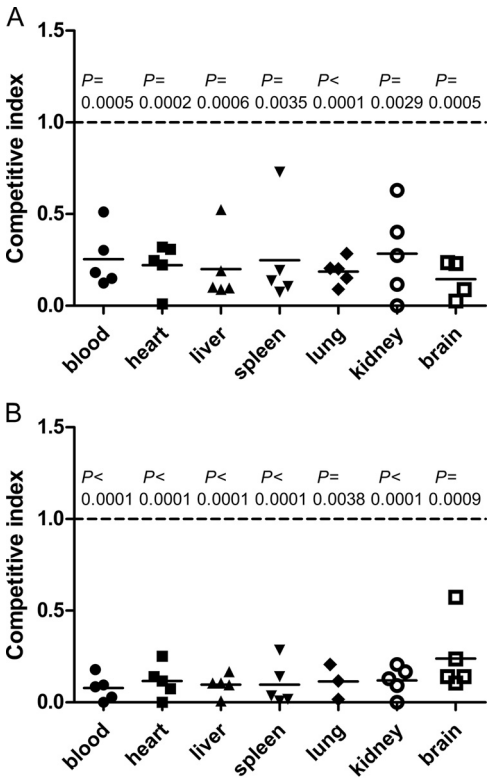

FIG 8 Competitive index of the Δ*troR* mutant against the WT strain (A) and CΔ*troR* complemented strain (B) in an intranasal mouse model. Groups of five BALB/c mice were intranasally infected with a 1:1 mixture of either the Δ*troR* mutant and the WT strain (group 1) or the Δ*troR* and CΔ*troR* strains (group 2). The blood, heart, liver, spleen, lung, kidney, and brain samples from the infected mice were collected at 12 h after infection. Bacteria recovered from the samples of mice in group 1 were analyzed by colony PCR to determine the competitive index (CI: the ratio of Δ*troR* mutant to WT in each sample divided by that in the inoculum). Bacteria recovered from the samples of mice in group 2 were analyzed for spectinomycin susceptibility to determine the CI (the ratio of Δ*troR* mutant to CΔ*troR* complemented strain in each sample divided by that in the inoculum). Mean CI values were compared to 1 (equal competitiveness) using the two-tailed paired *t* test to determine whether the difference in competitiveness was significant. As there were not enough bacterial cells recovered from the brain of one mouse in group 1, the data shown for this tissue are from four mice. As bacteria recovered from the lungs of two mice in group 2 were contaminated with other microbes, the data shown for this tissue are from three mice.

suggesting that the Δ*troR* mutant had reduced ability to colonize various tissues of mice in this model compared with the WT and CΔ*troR* strains. Thus, the virulence of the Δ*troR* mutant was significantly attenuated in the mouse intranasal infection model.

Taken together, TroR is required for *S. suis* virulence in an intranasal mouse model, despite the fact that it plays no apparent role in the virulence in the mouse intraperitoneal infection model.

DISCUSSION

As essential nutrients for life, metals play a pivotal role in the survival of bacteria within the host. Nevertheless, excess accumulation of intracellular metals is toxic to bacteria. Hence, it is not surprising that the vertebrate host develops strategies involving both metal limitation and metal excess to control bacterial infections (59). As a countermeasure, bacteria employ high-affinity metal transporters to compete with the host for metals and metal efflux pumps to export redundant metals (8). Typically, the genes encoding metal transporters and metal efflux pumps are controlled by metalloregulators, which can sense and bind metals (59). To date, the biological roles of metalloregulators in *S. suis* have received little attention. Only three metalloregulators (Zur, AdcR, and Fur) have been described in *S. suis* so far, yet their roles are not entirely elucidated (32, 36).

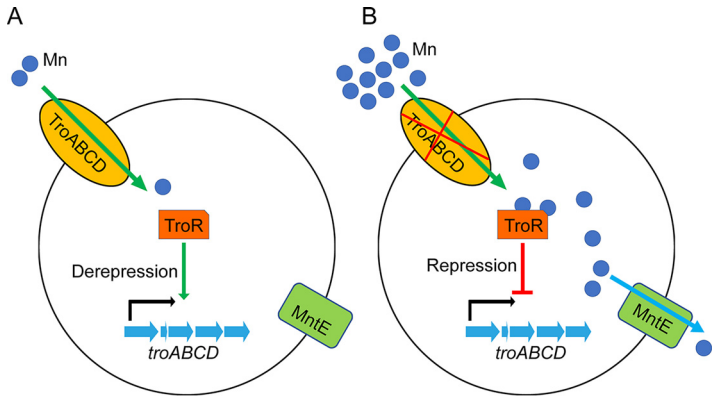

FIG 9 Proposed model for *S. suis* resistance to Mn toxicity. Under Mn limitation conditions (A), TroR derepresses the transcription of the *troABCD* operon, and the TroABCD system is expressed. Mn enters *S. suis* cells via the TroABCD system. Under Mn excess conditions (B), the expression of the TroABCD system is repressed by TroR, and Mn import is prevented. Moreover, the Mn efflux system MntE is activated; intracellular Mn can be exported outside the cells by MntE.

In the present study, we identified a metalloregulator, TroR, in *S. suis*. Both *troR* and the *troABCD* operon were significantly downregulated when *S. suis* was treated with Mn. A similar observation has been made for the homologous system (SloABCR) in *S. mutans* (60). In *S. mutans*, the binding of SloR to Mn promotes SloR dimerization and facilitates SloR binding to the promoter of the *sloABC* locus, thus blocking *sloABC* transcription, presumably via exclusion of RNA polymerase (61, 62). We speculated that in *S. suis*, Mn represses transcription of the *troABCD* operon via a similar mechanism. In addition, the promoter of *troR* is adjacent to the promoter of the *troABCD* operon in the genome of *S. suis*. We speculated that following Mn treatment, the binding of TroR to the promoter of *troABCD* affected RNA polymerase binding to the promoter of *troR*; thus, *troR* was also downregulated in the presence of excess Mn.

TroR is required for *S. suis* resistance to the toxicity conferred by Mn, Zn, Cu, and Co. The contribution of TroR homologs to Mn homeostasis has been well established in other species (11, 39, 53, 54). Similarly, we found that the Δ*troR* mutant exhibited decreased resistance to Mn toxicity when cultured under metal-depleted conditions. Although a higher level of intracellular Mn was accumulated in the Δ*troR* strain, it exhibited only a moderate growth defect when cultured in metal-replete medium supplemented with Mn. As observed in the present study and previous studies (10, 16), the toxicity of a metal may be rescued by another metal. The effect of Mn against the Δ*troR* strain in metal-replete medium may be rescued by trace amounts of other metals. RNA sequencing analysis revealed that the *troABCD* operon is repressed by TroR. Moreover, TroA has been shown to contribute to Mn acquisition in *S. suis* (33). Thus, it was not surprising that the Δ*troR* mutant, in which *troA* was significantly upregulated, accumulated a higher level of intracellular Mn than the WT and CΔ*troR* complemented strains. In a previous study, we demonstrated that the Mn efflux system MntE is involved in *S. suis* resistance to Mn toxicity (34). Taken together, *S. suis* could coordinate Mn import and export by the repression of the *troABCD* operon and activation of *mntE* in response to Mn toxicity (Fig. 9).

It has been well established that TroR homologs contribute to Mn homeostasis (11, 39, 53, 54). However, the involvement of TroR homologs in bacterial resistance to Zn, Cu, and Co toxicity has rarely been reported. It is unexpected that *S. suis* TroR contributes to Zn toxicity, as the levels of intracellular Zn in the WT, Δ*troR*, and CΔ*troR* strains were comparable following Zn treatment. Considering that *troA* is significantly upregulated in the Δ*troR* mutant and that TroA has the ability to bind Mn and Zn with nanomolar affinity (37), it is also confusing that the three strains could accumulate equal concentrations of Zn. In *S. pneumoniae*, Zn is not transported via the PsaABC system,

despite the fact that PsaA could bind Zn (63). Accordingly, we speculated that the TroABCD system of *S. suis* could not transport Zn; thus, the WT, Δ*troR*, and CΔ*troR* strains had similar accumulations of intracellular Zn. Consistent with this inference, *troA* expression in the WT strain was not significantly downregulated in response to Zn. Moreover, TroA exhibited no apparent role in Zn acquisition in a previous study (33). In *S. pneumoniae*, extracellular Zn can inhibit intracellular Mn accumulation with little effect on intracellular Zn (63). In *Escherichia coli*, Zn excess perturbs Fe and Cu homeostasis (64). It is reasonable to speculate that excess Zn results in disorder of metal homeostasis in *S. suis*. A possible explanation for the higher susceptibility of the Δ*troR* mutant to Zn is that deletion of *troR* reduces the ability of *S. suis* to respond to the disorder of metal homeostasis.

Similar to the case of Zn, Cu treatment resulted in a growth defect in the Δ*troR* strain, despite comparable Cu accumulation in the mutant. In *S. aureus*, a Δ*mntR* mutant accumulated increased levels of intracellular Cu and showed decreased growth in the presence of Cu (65). The observed growth defect of the mutant under Cu excess conditions is consistent with our result; however, we did not observe higher Cu accumulation in the Δ*troR* mutant. As *troA* expression was significantly downregulated when the WT strain was treated with Cu, it is reasonable to speculate that TroA can import Cu. The expression of *copA* (encoding a Cu efflux system) (35) was significantly upregulated in the Δ*troR* strain, revealing that the mutant suffered Cu stress. Taken together, we speculated that the Δ*troR* mutant indeed imports a higher level of Cu; however, the effect could be masked by the overexpression of the Cu efflux system. The Δ*troR* mutant also exhibited decreased resistance to Co toxicity. In line with this result, a higher level of intracellular Co was accumulated in the mutant. Moreover, the expression of the gene encoding PmtA, an Fe(II) and Co efflux pump (16), was upregulated in the Δ*troR* mutant.

Growth defects resulting from metal toxicity can often be attributed to mismetallation of metalloproteins with nonactivating metals (59, 66). In certain bacteria, the deletion of the efflux pump for a metal has been found to result in a growth defect in the presence of that metal, which can usually be alleviated by the addition of another metal (10, 16, 67, 68). In line with these findings, we showed that the growth defect of the Δ*troR* mutant under Zn and Co excess conditions could be partly rescued by Mn supplementation. However, the growth inhibition of the Δ*troR* strain resulting from excess Cu was not alleviated by Mn supplementation. Moreover, the growth of the WT and CΔ*troR* complemented strains was severely inhibited following Mn supplementation. According to the Irving-Williams stability series on protein-metal affinity [Mg/Ca $<$ Mn $<$ Fe(II) $<$ Co $<$ Ni $<$ Cu $>$ Zn] (69), Cu has the highest affinity to proteins. Hence, we speculated that if metalloproteins are mismetallated by Cu, Mn would not have the advantage of competing with Cu; instead, Mn itself would exert a toxic effect. The growth of the Δ*troR* mutant was severely inhibited by Cu. Hence, Mn supplementation did not enhance the growth defect of the Δ*troR* strain. Nevertheless, the WT and CΔ*troR* strains grew much better than the Δ*troR* mutant in the presence of Cu; thus, the toxic effect should be more prominent.

The regulon of TroR homologs appears to be species and strain specific. In *S. suis*, TroR repressed the expression of the *troABCD* operon. This result is consistent with previous observations in several species, such as *S. pneumoniae* and *S. pyogenes* serotypes M1 and M3 (39, 44, 70). Nevertheless, *mtsA* transcripts were not changed in the *mtsR* mutant of *S. pyogenes* serotype M49 (40). In *S. pyogenes* serotype M49, an increase in the transcription of genes encoding the metal exporters PmtA and CopA was observed in the *mtsR* mutant (40). In line with this result, the expression of *pmtA* and *copA* was significantly upregulated in the Δ*troR* mutant of *S. suis*. The gene encoding serine protease was the most upregulated gene in the Δ*troR* mutant. Similarly, the homologous gene in *S. pneumoniae* was significantly upregulated in a Δ*psaR* mutant (56). In addition, we found that in *S. suis*, *troA* expression was upregulated in response to Fe(II) and Co and downregulated in response to Mn and Cu. The effect of metal treatment on the

expression of *troA* homologs has been well characterized in *S. pneumoniae* (42–45). While Mn negatively affects the expression of *psaBCA*, Zn, Co, and Ni have the opposite effect on the expression of this operon (42–45). The impact of Mn and Co on *troA* expression is consistent with the findings in *S. pneumoniae* (44, 45); however, we did not observe any role of Zn and Ni in *troA* expression. According to the mechanisms of metal sensing and regulation by TroR homologs in *S. pyogenes* and *S. mutans* (39, 61, 62), we speculated that Fe(II) and Co could compete with Mn for binding to TroR, affecting TroR binding to the promoter of *troABCD*; thus, *troA* was upregulated in the presence of excess Fe(II) and Co. Interestingly, *troA* expression was significantly repressed by Cu, indicating that TroA plays a potential role in Cu import, which has also been suggested by another study (65). Since Cu represses *troA* expression even in the Δ*troR* mutant, it is likely that other regulators, such as CopY, which is involved in Cu homeostasis (36), might also participate in regulating the *troABCD* operon.

TroR homologs have been shown to be required for the virulence of certain pathogens (39, 44, 56–58). While no apparent role was observed for TroR in *S. suis* virulence in a mouse intraperitoneal infection model, the Δ*troR* mutant had a significantly reduced ability to colonize various tissues of mice in an intranasal mouse model. It is possible that the metal contents are very low in the abdominal cavity of mice; the bacteria might not suffer from metal toxicity when they were inoculated by intraperitoneal injection. The intranasal mouse model is another model widely used for studying the virulence of *S. suis* (71–73). A well-established competitive-infection assay (74–76) with this model was further performed to compare the virulence of the Δ*troR* mutant with that of the WT and CΔ*troR* complemented strains. The data clearly showed that the Δ*troR* strain had reduced ability to colonize various tissues of mice, revealing that the virulence of the Δ*troR* strain was attenuated in the intranasal mouse model. It has been demonstrated that mice could increase metal concentrations in various tissues, such as nasopharynx and lung, to respond to intranasal infection with *S. pneumoniae* (63). Accordingly, we speculated that the levels of metals such as Zn were increased in the nasopharynx of mice in response to *S. suis* infection. Therefore, the Δ*troR* mutant, which was defective in resistance to metal toxicity, exhibited attenuated virulence in the intranasal mouse model.

While some interesting findings have been reported, several questions still need to be answered. Although TroR contributes to *S. suis* resistance to Zn and Cu toxicity, the real mechanisms remain unknown. The Δ*troR* mutant accumulates higher levels of intracellular Co; however, little is known about how Co enters *S. suis* cells and why the WT and Δ*troR* strains uptake different levels of Co. As the gene encoding serine protease was significantly differentially expressed in response to Mn, Fe(II), and Co (55), and it was the most upregulated gene in the Δ*troR* strain, evaluation of the roles of serine protease in metal homeostasis would be interesting. In *S. pneumoniae*, PsaR has a strain-specific impact on global gene expression and bacterial virulence (56). Further studies can be performed to examine the roles of TroR and the TroABCD system in other serotypes of *S. suis*. Although TroR homologs generally contribute to the homeostasis of one or two metals (38), *S. suis* TroR plays a role in resistance to the toxicity conferred by multiple metals. A structural basis for metal sensing by TroR is also needed to elucidate the mechanism underlying its function.

In conclusion, the expression of the *troABCD* operon was significantly downregulated when *S. suis* was treated with excess Mn. TroR, a metalloregulator that potentially represses the *troABCD* operon, was hypothesized to play a role in the maintenance of Mn and Zn homeostasis. Functional analysis of TroR revealed that it represses expression of the *troABCD* operon and contributes to the resistance of *S. suis* to the toxicity conferred by Mn, Zn, Cu, and Co. TroR is required for *S. suis* virulence in the mouse intranasal infection model, despite the fact that no apparent role in the virulence was observed in the mouse intraperitoneal infection model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2; the primers are listed in Table S2 in the supplemental material. Unless otherwise

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
SC19	Virulent *S. suis* strain isolated from brain of dead pig	Laboratory collection
Δ*troR*	*troR* deletion mutant of strain SC19	This study
CΔ*troR*	Complemented strain of Δ*troR* mutant; Spc^r	This study
DH5α	Cloning host for recombinant vector	Tsingke
Plasmids		
pSET4s	Thermosensitive suicide vector; Spc^r	77
pSET4s-Δ*troR*	Knockout vector for *troR* deletion	This study
pSET2	*E. coli-S. suis* shuttle vector; Spc^r	79
pSET2-*troR*	pSET2 containing *troR* and its promoter	This study

[a]Spc^r, spectinomycin resistant.

specified, *S. suis* strains were cultured at 37°C in tryptic soy broth (Becton, Dickinson and Company) supplemented with 10% (vol/vol) newborn bovine serum (Sijiqing, Hangzhou, China) (TSBS [i.e., metal-replete medium]) or on tryptic soy agar (Becton, Dickinson and Company) supplemented with 10% (vol/vol) newborn bovine serum (TSAS). *E. coli* strains were cultured in Luria-Bertani (LB) broth or on LB agar. When required, spectinomycin was added to the medium at 100 μg/ml for *S. suis* or 50 μg/ml for *E. coli*. Metal-deplete medium was prepared by incubating TSBS with 1% Chelex-100 resin (Sigma-Aldrich) with rotation for 18 h. The metal salts (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade and were dissolved in deionized water to prepare the metal stock, except for Fe(II). As Fe(II) is easily oxidized, Fe(II) solution was prepared before each use.

RNA extraction. To explore the transcriptomic change in *S. suis* in response to Mn, the *S. suis* 2 strain SC19 was grown in TSBS medium to the mid-exponential phase (OD_{600} of 0.6) and divided into two equal aliquots: one aliquot was supplemented with 1 mM Mn and the other with deionized water. The cultures were incubated for another 15 min and then centrifuged for collecting bacterial cells. The cell pellets were immediately subjected to RNA extraction using the Eastep Super total RNA extraction kit (Promega, Shanghai, China). The RNA concentration and integrity were determined using the Qubit 2.0 fluorometer and the Agilent 2100 Bioanalyzer system, respectively. Three independent experiments were performed to obtain biological triplicate samples.

To compare the differences in the transcriptomes of the WT strain and Δ*troR* mutant, the two strains were grown in TSBS medium to an OD_{600} of 0.6; bacterial cells were collected by centrifugation and then subjected to RNA extraction. RNA extraction and assessment were performed as described above. Three independent experiments were performed to obtain biological triplicate samples.

To evaluate *troA* expression in the WT strain and Δ*troR* mutant in response to various metals, both strains were grown in TSBS medium to an OD_{600} of 0.6; each culture was then divided into nine equal aliquots, which were supplemented with 2 mM Fe(II), 2 mM Mn, 50 μM Zn, 1 mM Cu, 1 mM Co, 1 mM Ni, 2 mM Ca, 2 mM Mg, or deionized water. After 15 min of incubation, the bacterial cells were collected for RNA extraction. RNA extraction and assessment were performed as described above. Three independent experiments were performed to obtain biological triplicate samples.

RNA sequencing analysis. RNA sequencing analysis was performed with the help of Novogene Bioinformatics Technology Co., Ltd. cDNA library preparation, sequencing, and data analysis were performed as described previously (55).

Mutant construction and functional complementation. The Δ*troR* mutant was constructed in the *S. suis* 2 strain SC19 background using the pSET4s suicide plasmid (77), following previously described procedures (78). The CΔ*troR* complemented strain was generated using the pSET2 plasmid (79), as described previously (80).

Growth curve analyses. The WT, Δ*troR*, and CΔ*troR* strains were grown in TSBS medium to the early stationary phase (OD_{600} of 1.2) and then diluted in fresh medium (TSBS or Chelex-treated TSBS) supplemented with various concentrations of the specified metals. In the medium supplemented with Fe(II), 1 g/liter of trisodium citrate dihydrate was added to reduce Fe precipitation (67). The cultures were subpacked in 96-well plates (200 μl/well, three wells per culture). The plates were incubated at 37°C with linear shaking (120 rpm), and the OD_{595} values were measured hourly using the CMax Plus plate reader (Molecular Devices, Shanghai, China). At least three independent experiments were performed for each condition.

Intracellular metal content measurement. The WT, Δ*troR*, and CΔ*troR* strains were grown in TSBS medium to an OD_{600} of 0.6 and then supplemented with metals (4 mM Mn, 100 μM Zn, 1.5 mM Cu, or 1 mM Co). The cultures were incubated for another 30 min and then centrifuged for collection of bacterial cells. The bacterial cells were washed three times with PBS containing 0.25 M EDTA and three times with PBS alone. After drying at 110°C, the cells were weighed, digested in 66% nitric acid at 70°C for 48 h, and diluted to 2% nitric acid with deionized water. Following this, the metal concentration in the cells was analyzed by ICP-OES. The metal content was expressed as μg of metal per g of cells (dry weight). Four independent experiments were performed to obtain four biological samples for each strain and each condition.

Reverse transcription-quantitative PCR analysis. Approximately 0.2 μg of RNA per sample was converted to cDNA using the NovoScript Plus All-in-One 1st Strand cDNA synthesis SuperMix (gDNA Purge; Novoprotein, Shanghai, China). Quantitative PCR was performed on the StepOnePlus real-time PCR system (Applied Biosystems) using NovoStart SYBR qPCR SuperMix Plus (Novoprotein, Shanghai, China) and the primers specified in Table S2. The relative gene expression level was analyzed using the threshold cycle ($2^{-\Delta\Delta CT}$) method, with 16S rRNA being used the reference gene (81). The experiments were performed in triplicate using biological triplicate samples.

Virulence study using murine infection models. The animal studies were approved by the Animal Welfare and Ethics Committees of Yangzhou University. In total, 50 female BALB/c mice (4 to 6 weeks old) were used for the virulence study.

For the mouse intraperitoneal infection model, 40 BALB/c mice were randomly divided into four groups (10 mice per group). The mice in groups 1, 2, and 3 were intraperitoneally infected with 3×10^8 CFU of the WT, Δ*troR*, and CΔ*troR* strains, respectively. The mice in group 4 were intraperitoneally infected with 300 μl PBS, and served as the control. The clinical symptoms and survival rates were recorded twice daily for 7 days.

For the competitive-infection assay with the mouse intranasal infection model, 10 BALB/c mice were randomly divided into two groups (five mice per group). The mice were anesthetized via inhalation of isoflurane and pretreated with 12.5 μl of 1% acetic acid placed in each nostril 1 h prior to intranasal infection. After controlled recovery and additional anesthesia, the mice in group 1 were intranasally infected with a 1:1 mixture of the Δ*troR* and WT strains (20 μl in total, 2×10^8 CFU for each strain), and the mice in group 2 were infected with a 1:1 mixture of the Δ*troR* and CΔ*troR* strains (20 μl in total, 2×10^8 CFU for each strain). At 12 h after infection, the blood, heart, liver, spleen, lung, kidney, and brain samples from the infected mice were collected. The blood samples were diluted and plated, while the other samples were homogenized in 1 ml PBS, diluted, and plated. For each sample in group 1, a total of 70 to 80 colonies were analyzed by colony PCR with the primer pair Out1/Out2 to determine the CI. For each sample in group 2, a total of 70 to 80 colonies were analyzed for spectinomycin susceptibility (simultaneously transferred to plates with and without spectinomycin) to determine the CI.

Bioinformatic analysis. Protein sequences of the DtxR family metalloregulators were obtained by searching the NCBI database using their GenBank accession numbers or locus tags found in the articles cited. The sequences were submitted to Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to generate the phylogenetic tree, which was further edited using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). The BPROM program (http://linux1.softberry.com/berry.phtml) was used to predict the promoter of *troR*.

Statistical analysis. GraphPad Prism 5 was used for data analysis. The differences in metal content were analyzed with the two-tailed unpaired *t* test. *troA* expression in the WT strain in response to various metals was analyzed by one-way analysis of variance with Bonferroni's posttest. Two-way analysis of variance with Bonferroni's posttest was used for comparison of *troA* expression in the Δ*troR* mutant with that in the WT strain. The two-tailed paired *t* test was used to analyze the data in the competitive-infection assay. The log rank test was used to analyze the murine survival rates.

Data availability. RNA sequencing data have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession no. GSE164206 and GSE164207.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

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