



PerR-Regulated Manganese Import Contributes to Oxidative Stress Defense in *Streptococcus suis*

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ABSTRACT *Streptococcus suis* has been increasingly recognized as a porcine zoonotic pathogen that threatens the health of both pigs and humans. Metal homeostasis plays a critical role in the antioxidative capability of bacteria, thus facilitating the escape of pathogenic species from the innate immunity systems of hosts. Here, we revealed that manganese increased the ability of *S. suis* to resist oxidative stress. RNA sequencing was used to identify potential candidate genes involved in the maintenance of intracellular manganese homeostasis. Four genes, termed *troABCD*, were identified by NCBI BLASTp analysis. The *troA*, *troB*, *troC*, and *troD* deletion mutant strains exhibited decreased intracellular manganese content and tolerance to H₂O₂ compared to the wild-type strain. Thus, *troABCD* were determined to be involved in manganese uptake and played an important role in H₂O₂ tolerance in *S. suis*. Furthermore, the inactivation of *perR* increased the survival of H₂O₂-pulsed *S. suis* 2.18-fold and elevated the intracellular manganese content. H₂O₂-pulsed *S. suis* and *perR* deletion mutants upregulated *troABCD*. This finding suggested that H₂O₂ released the suppression of *troABCD* by *perR*. In addition, an electrophoretic mobility shift assay (EMSA) showed that PerR at 500 ng binds to the *troABCD* promoter, indicating that *troABCD* were directly regulated by PerR. In conclusion, this study revealed that manganese increases tolerance to H₂O₂ by upregulating the expression of *troABCD*. Moreover, PerR-regulated Mn import in *S. suis* and increased the tolerance of *S. suis* to oxidative stress by regulating *troABCD*.

IMPORTANCE During infection, it is extremely important for bacteria to defend against oxidative stress. While manganese plays an important role in this process, its role is unclear in *S. suis*. Here, we demonstrated that manganese increased *S. suis* tolerance to oxidative stress. Four manganese ABC transporter genes, *troABCD*, were identified. Oxidative stress increased the content of manganese in the cell. Furthermore, PerR increased the tolerance to oxidative stress of *S. suis* by regulating *troABCD*. Manganese played an important role in bacterial defense against oxidative stress. These findings provide novel insight into the mechanism by which *S. suis* resists oxidative stress and approaches to inhibit bacterial infection by limiting manganese intake.

KEYWORDS *Streptococcus suis*, manganese, *troABCD*, oxidative stress, PerR

Bacteria encounter oxidative stress when exposed to reactive oxygen species (ROS) and have consequently developed various mechanisms of protection (1, 2). ROS, including superoxide ion (O₂⁻), hydroxyl radical (HO·), and hydrogen peroxide (H₂O₂), can cause severe damage to lipids, proteins, and DNA (3). Multiple factors, including

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superoxide dismutase (SOD), NADH oxidase, catalase, and DNA-binding protein, from starved cells play an important role in tolerance to oxidative stress (4, 5). Although H_2O_2 is not particularly toxic to microorganisms, it can react with cellular Fe^{2+} via Fenton chemistry to form highly toxic $HO\cdot$ (6).

The most well-known effect of manganese is related to its involvement in oxidative stress defense mechanisms (7). Manganese is an essential cofactor of manganese-containing SOD (8) and also acts as an inorganic enzyme to dismutate O_2^- by complexation with small molecules of bacteria (9). The host manganese chelator calprotectin and the deletion of manganese importers have been shown to diminish SOD activity in *Staphylococcus aureus* (10, 11). Manganese inhibits ferrooxidation and prevents damage to Dps-1-bound DNA derived from iron leakage from the core, followed by the generation of $\cdot OH$ through Fenton chemistry (12). Additionally, manganese functions as an antioxidant via a protein-independent mechanism (9).

A Fur family regulator, PerR, has been identified in various bacteria and is associated with significant variability in bacterial sensitivity to oxidation (13). PerR is associated with the suppression of oxidative stress detoxification genes, including *sodA*, *dpr*, *pmtA*, and *ahpCF* (14–17). There are various compositions of regulons governed by PerR among bacterial species and even between strains within a species. The genes regulated by PerR can be divided into two major groups: (i) genes involved in metal homeostasis and (ii) genes directly involved in ROS detoxification (13). PerR has been reported to be involved in the oxidative stress response in *Streptococcus pyogenes* (16), *Streptococcus oligofermentans* (18), and *Streptococcus mutans* (19). Moreover, *Streptococcus suis* is an increasingly recognized porcine zoonotic pathogen responsible for huge economic losses to the pig industry (20). Of the 29 known serotypes (21), *S. suis* serotype 2 is considered to be the most pathogenic. Two large-scale outbreaks of *S. suis* occurred in China in 1998 and 2005 (22, 23). *S. suis* can cause a variety of severe infections, including arthritis, meningitis, endocarditis, septicemia, and even death, in both swine and humans (21). It is extremely important for pathogenic bacteria to adapt to the host environment and develop defenses against oxidative stress (24). The regulation of responses to oxidative stress is extremely important for bacterial survival *in vivo* when bacteria encounter oxidative stress during infection. PerR has been demonstrated to regulate manganese uptake and defend against oxidative stress in *Streptococcus oligofermentans* (18). However, in *S. suis*, PerR has been reported only to regulate the oxidative stress response through the regulation of *dpr* (25). In addition, the relationship between PerR and manganese uptake is poorly understood. Furthermore, the role of manganese in *S. suis* tolerance to oxidative stress and how manganese uptake responds to oxidative stress remain unclear. In this study, we revealed the importance of manganese in *S. suis* H_2O_2 tolerance. In addition, PerR derepressed the manganese transporter *troABCD* genes in the presence of H_2O_2 .

RESULTS

Manganese increases the ability of *S. suis* to tolerate oxidative stress. Manganese has been illustrated to increase the tolerance of *Streptococcus oligofermentans* (18), *Staphylococcus aureus* (26), and *Deinococcus radiodurans* (27) to oxidative stress. To explore the role of cellular manganese in oxidative stress tolerance, mid-log-phase cultures of *S. suis* were collected and spotted onto tryptic soy agar (TSA) plates supplemented with H_2O_2 or $MnCl_2$ at different concentrations. As shown in Fig. 1, no colonies formed from 10 mM H_2O_2 , and with the addition of 0.25 to 0.5 mM Mn^{2+} , colonies formed in the presence of 10 mM H_2O_2 . These results revealed that manganese played an important role in *S. suis* resistance to H_2O_2 .

Transcriptional response of *S. suis* to excess exogenous manganese. To identify potential candidate genes involved in the maintenance of intracellular manganese homeostasis, RNA sequencing (RNA-seq) was used to investigate the transcriptional response of *S. suis* exposed to 1 mM $MnCl_2$ compared to untreated cells (Fig. 2).

Four transcripts (SSUSC84_1887, SSUSC84_1888, SSUSC84_1889, and SSUSC84_1891), representing the most downregulated genes encoding metal ABC transporters, exhibited 3.9- to 22.45-fold decreases in transcript abundance in cells exposed to 1 mM Mn. SSUSC84_1891

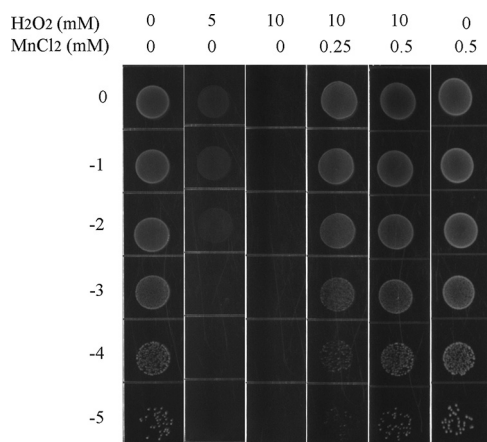


FIG 1 Spot dilution assays of the wild-type strain with or without H₂O₂ or manganese.

has been reported previously in *S. suis* as *troA* (28). SSUSC84_1889 exhibited approximately 41.77%, 40.17%, 41.35%, 39.66%, and 41.83% amino acid identities to the manganese transporters PsaA from *Streptococcus pneumoniae*, MtsA from *Streptococcus pyogenes*, MntA from *Streptococcus oligofermentans*, MntA from *Staphylococcus aureus*, and EfaA from *Enterococcus faecalis*, respectively (see Fig. S1 in the supplemental material). Therefore, SSUSC84_1887, SSUSC84_1888, SSUSC84_1889, and SSUSC84_1891 were designated

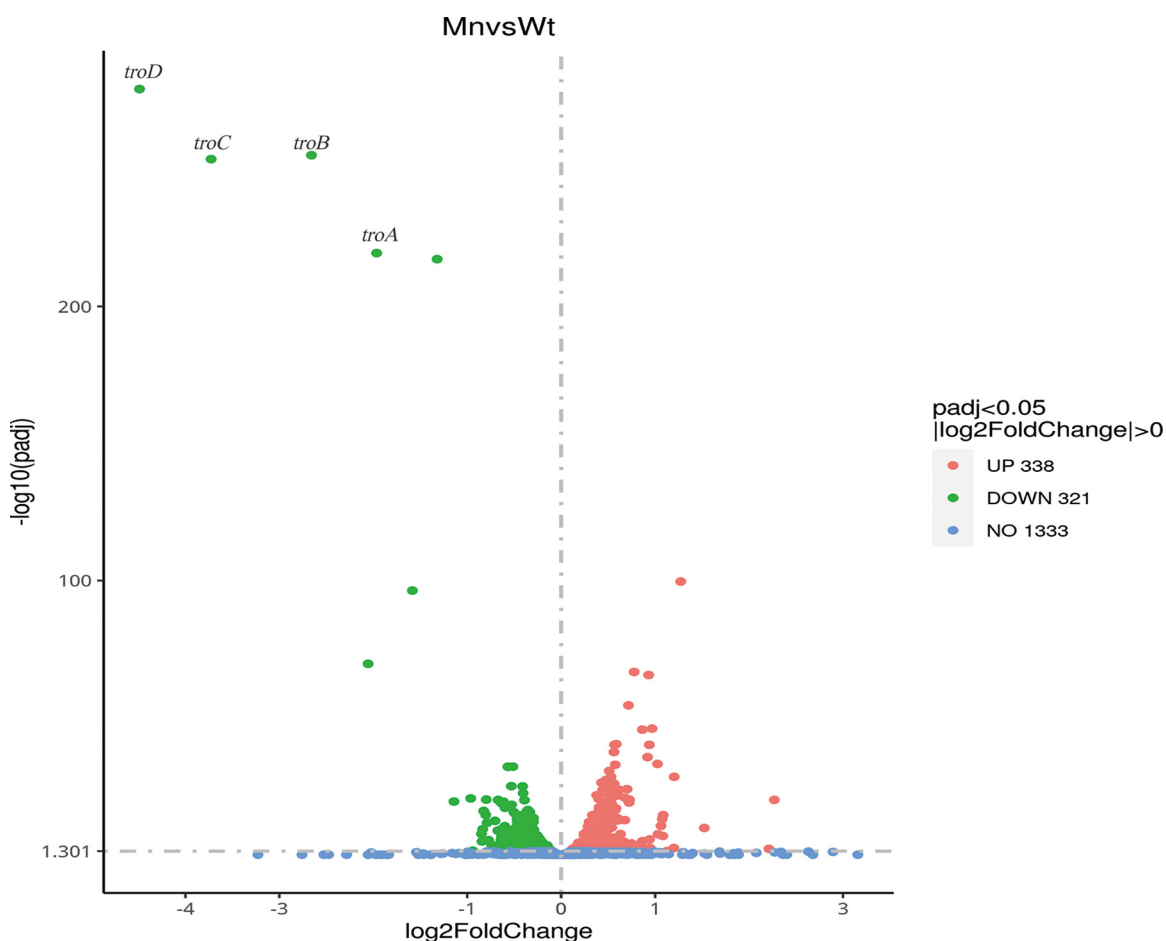


FIG 2 Exposure to 1 mM MnCl₂ alters the *S. suis* transcriptome. Shown are results from RNA sequencing analysis measuring fold changes in transcript abundances for *S. suis* wild-type cells treated with 1 mM MnCl₂ compared to untreated controls.

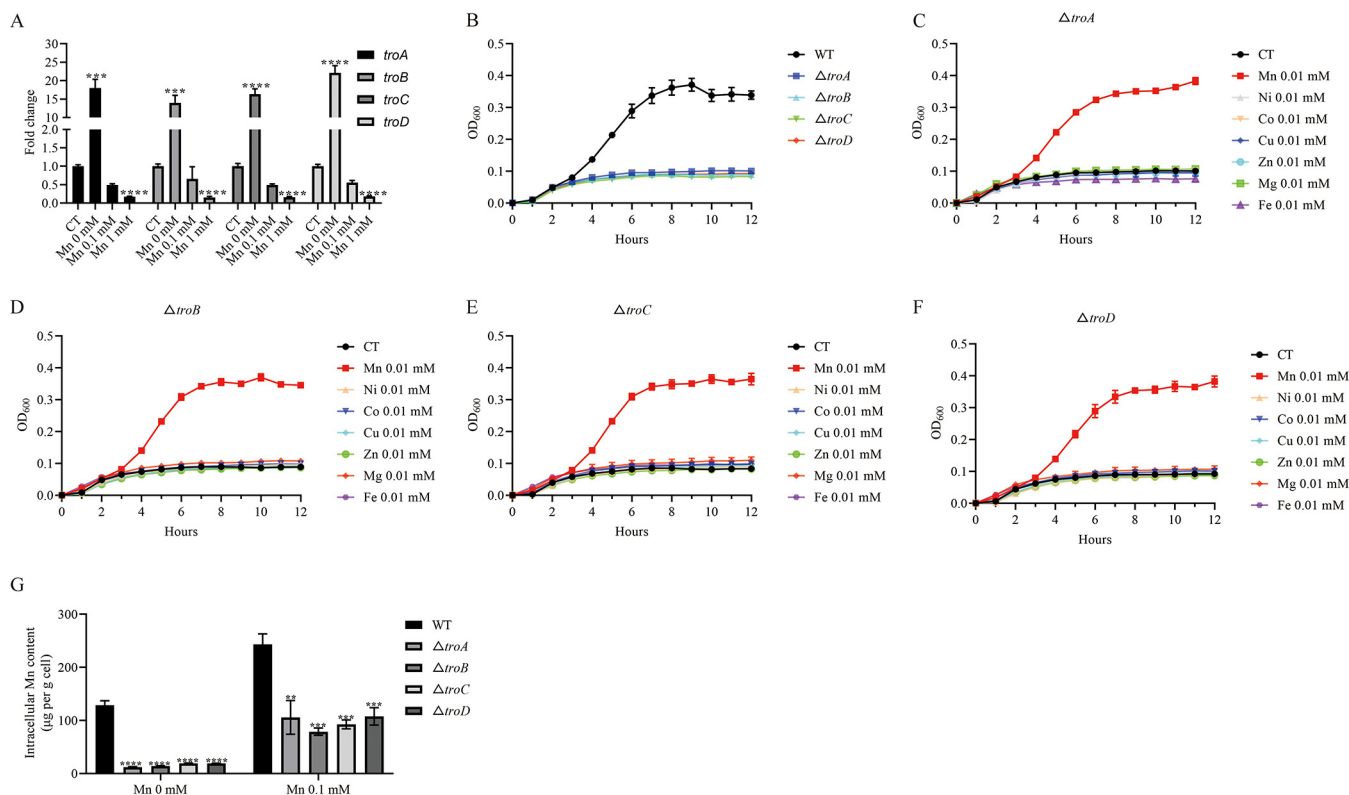


FIG 3 TroABCD promotes the growth of *S. suis* in Mn-restricted environments. (A) Transcription levels of *troA*, *troB*, *troC*, and *troD* of *S. suis* in CDM supplemented with or without Mn. (B) Growth curves of the WT, $\Delta troA$, $\Delta troB$, $\Delta troC$, and $\Delta troD$ strains in CDM without Mn. (C to F) Growth curves of the $\Delta troA$, $\Delta troB$, $\Delta troC$, and $\Delta troD$ strains in CDM with or without metal ions. (G) Intracellular Mn contents of the WT, $\Delta troA$, $\Delta troB$, $\Delta troC$, and $\Delta troD$ strains treated with or without 0.1 mM Mn. The data are expressed as means \pm standard deviations from three independent experiments. Asterisks indicate *P* values of <0.05 by Student's *t* tests on the samples. CT, control; OD₆₀₀, optical density at 600 nm.

troABCD and inferred to be involved in manganese transport.

TroABCD promotes *S. suis* growth in Mn-restricted environments and functions as a manganese transporter. To explore the role of TroABCD in Mn environments, we examined the transcription levels of *troA*, *troB*, *troC*, and *troD* in chemically defined medium (CDM) (29) with or without Mn. As shown in Fig. 3A, the transcription levels of *troA*, *troB*, *troC*, and *troD* were significantly higher (14- to 22.12-fold) in CDM without Mn than in CDM containing Mn. Furthermore, the transcription levels decreased with higher concentrations of Mn (Fig. 3A). Next, we generated $\Delta troA$, $\Delta troB$, $\Delta troC$, and $\Delta troD$ mutant strains using a markerless system (see Fig. S2 in the supplemental material). Upon genetic confirmation that all gene deletions occurred as planned, the growth of wild-type (WT) and mutant strains was monitored in Mn-depleted CDM. None of the mutant strains could grow in Mn-deficient medium, whereas the WT strain grew normally (Fig. 3B). The mutant strains could grow when supplemented with Mn but not other metal ions (Ni, Cu, Co, Zn, Mg, and Fe) (Fig. 3C to F). Collectively, these results demonstrated that the transcription levels of *troABCD* increased in Mn-restricted environments and that TroABCD contributed to the growth of *S. suis* in Mn-restricted environments.

To verify the primary function of the ABC transporter system in the process of manganese transport, we used a flame atomic absorption spectrophotometer to quantify the cellular Mn content in each of the different Mn transport mutants. As shown in Fig. 3G, mutations in the *troABCD* components caused a significant reduction in the intracellular Mn pool, with or without supplementary Mn. These results demonstrated that TroABCD was the main Mn importer in *S. suis*. The Mn uptake system promoted *S. suis* growth in Mn-restricted environments.

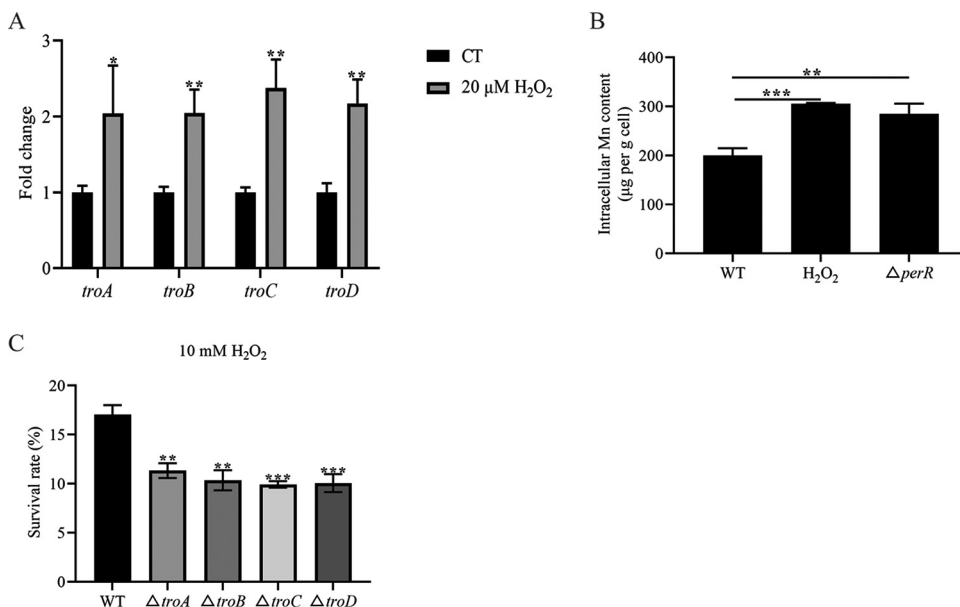


FIG 4 TroABCD contributed to the resistance to H₂O₂ of *S. suis*. (A) Transcription levels of *troABCD* of *S. suis* treated with or without H₂O₂. (B) Intracellular Mn content. (C) Survival rates of WT and deletion mutant strains treated with 10 mM H₂O₂. The data are expressed as means ± standard deviations from three independent experiments. Asterisks indicate *P* values of <0.05 by Student's *t* tests on the samples.

TroABCD contributes to *S. suis* resistance to H₂O₂. The transcription of *mntABC* has been reported to be induced by H₂O₂ and to play an important role in H₂O₂ resistance (30). To investigate the role of TroABCD in *S. suis* resistance to H₂O₂, the WT strain was cultured with 0 or 20 μM H₂O₂ to mid-log phase. The RNA expression of *troABCD* was measured, and as shown in Fig. 4A, the *troABCD* expression level was higher in 20 μM H₂O₂ than the control one. The intracellular Mn content of *S. suis* treated with H₂O₂ was increased compared to the control (Fig. 4B).

Furthermore, *troABCD* deletion mutant strains were used to evaluate H₂O₂ tolerance. As shown in Fig. 4C, the WT and deletion mutant strains were cultured with or without H₂O₂, and the survival rates of the deletion mutant strains were low compared to that of the WT strain. These data suggested that the manganese transporter TroABCD played an important role in the H₂O₂ tolerance of *S. suis*.

The *perR* deletion increased tolerance to H₂O₂ and the cellular manganese content. PerR was indicated to be a peroxide-responsive repressor in *S. suis* (25). To identify the function of PerR in *S. suis*, a *perR* deletion mutant was constructed and verified by PCR (Fig. S1B) and sequencing. The WT strain, the *perR* deletion mutant (Δ*perR*), and the complementation strain (CΔ*perR*) were cultured with or without H₂O₂. As shown in Fig. 5A, the survival of the Δ*perR* mutant was 2.18-fold higher than that of the WT strain. PerR has been reported to regulate metal ion homeostasis in *Bacillus subtilis* (31) and to downregulate cellular manganese in *S. oligofermentans* (18). To explore whether PerR played a similar role in *S. suis*, cells in the mid-log phase were collected to measure the levels of cellular manganese using a flame atomic absorption spectrophotometer. As shown in Fig. 4B, the manganese content was higher in the Δ*perR* mutant than in the WT and CΔ*perR* strains. These results revealed that PerR downregulates cellular manganese in *S. suis*.

PerR inactivation upregulates *troABCD*, and PerR binds to the *troABCD* promoter. Because the level of cellular manganese was increased in the Δ*perR* mutant, *troABCD* expression was evaluated. As shown in Fig. 5B, the expression of *troABCD* in the Δ*perR* mutant was upregulated compared to the WT strain. Furthermore, in the *perR* mutant, the transcription levels of *troABCD* were not increased following treatment with H₂O₂ (Fig. 5C). To explore whether PerR-regulated *troABCD* indirectly or directly, we assessed PerR binding to the promoter region of *troA* using an electrophoretic mobility shift

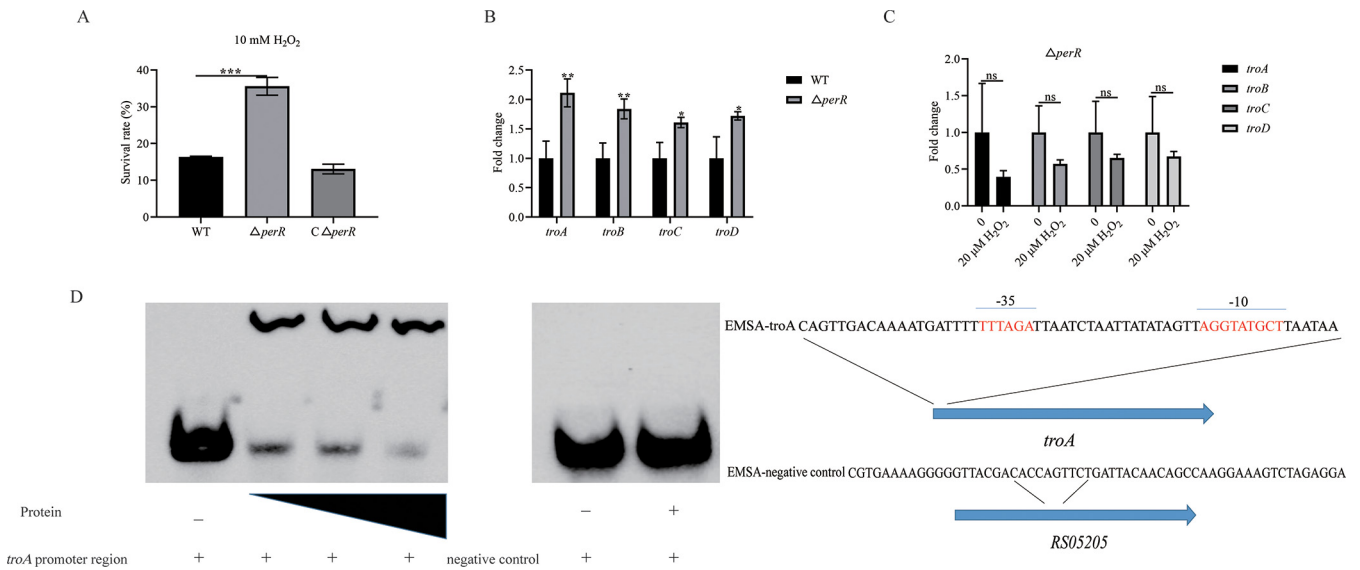


FIG 5 PerR played an important role in resistance to H₂O₂ and regulating *troABCD*. (A) Survival rates of WT and deletion mutant strains treated with 10 mM H₂O₂. (B) Transcription levels of *troABCD* of *S. suis* or the $\Delta perR$ mutant. (C) Transcription levels of *troABCD* in the *perR* mutant treated with or without H₂O₂. (D) An EMSA was performed to evaluate the direct binding of PerR to the promoter region of *troABCD*. The left panel shows PerR (0 ng, 500 ng, 600 ng, and 700 ng) and a biotin-labeled DNA probe. The right panel shows PerR (700 ng) and a nonspecific DNA probe. The locations of the two probes in the genome are also shown. The data are expressed as means \pm standard deviations from three independent experiments. Asterisks indicate *P* values of <0.05 by Student's *t* tests on the samples. ns, not significant.

assay (EMSA). As shown in Fig. 5D, PerR bound directly to the *troABCD* promoter region but was not able to bind to a nonspecific DNA fragment (Fig. 5D).

These results demonstrated that PerR represses the expression of *troABCD* and that H₂O₂ released the repression. Moreover, PerR increased *S. suis* tolerance to H₂O₂ by increasing the expression of *troABCD*.

DISCUSSION

Most streptococci are commensals, pathogens, or opportunistic pathogens of animals and humans (32). During the process of infection, bacteria may encounter situations of both metal starvation and oxidative stress (33, 34). For pathogenic bacteria, adaptation to the host environment is crucial for their survival and the establishment of a successful infection (24). During host-pathogen interactions, neutrophils use an "oxidative burst" to kill pathogenic bacteria, which is extremely important for bacteria to resist oxidative stress (35, 36). Insufficient intracellular manganese can have wide-ranging consequences, affecting the Mn-dependent enzymes involved in transcription, metabolism, and defense against oxidative stress (26, 37, 38). Mn is the most well-known metal ion involved in antioxidant stress, which increases the antioxidant capacity of bacteria through a variety of mechanisms (7). In this study, we report that *S. suis* could resist H₂O₂ by regulating the import of Mn through PerR.

Transition metals are essential trace metal elements for bacteria. Lactic acid bacteria (e.g., streptococci) are notoriously Mn-centric organisms, exhibiting a much higher nutritional demand for Mn than other bacterial groups (39). This particular metabolic phenomenon might explain why the striking loss of virulence observed here is in contrast to the moderate virulence attenuation of Mn transport mutants in Gram-negative and other Gram-positive pathogens (10, 40). In this work, *S. suis* increased the uptake of manganese in the presence of H₂O₂ and the ability to tolerate H₂O₂ by supplementing manganese (Fig. 1 and Fig. 4B).

To explore the genes involved in *S. suis* manganese regulation, RNA-seq was used to analyze the levels of RNA expression. Four genes displayed a large degree of down-regulation when the WT strain was treated with manganese. Using amino acid sequence analysis, the genes had high homology with *mntABC* of other streptococci.

In addition, the manganese ABC transporter is the primary transporter for bacterial uptake of manganese, which has been reported in *Staphylococcus aureus* (41) and *Bacillus anthracis* (42). It has been identified to affect the oxidative stress resistance of *Streptococcus gordonii* (18, 43). However, the role of TroABCD has seldom been reported in *S. suis*. To investigate the role of *troABCD* in resistance to H₂O₂, we constructed deletions of *troA*, *troB*, *troC*, and *troD*. The deletion mutant strains decreased intracellular manganese and attenuated H₂O₂-challenged survival. Furthermore, the *troABCD* expression levels were increased in the WT strain in response to treatment with H₂O₂ (Fig. 4A). These findings indicated that *troABCD*-mediated manganese uptake was pivotal to antioxidative stress tolerance in *S. suis*.

PerR has been reported to regulate the oxidative stress response by increasing *dpr* expression in *S. suis* (25). Because Dpr has iron storage properties, cytosolic iron can be efficiently scavenged when *dpr* is overexpressed in *S. suis* (44). To determine whether PerR was involved in manganese uptake, the mRNA expression of *troABCD* was detected in Δ *perR*. The expression of *troABCD* was increased in the *perR* deletion mutant strain compared with that of the WT strain (Fig. 5B). Furthermore, the intracellular manganese content of the Δ *perR* mutant was higher than that of the WT strain. In the presence of endogenous or exogenous H₂O₂, PerR derepressed the expression of the manganese transporter genes *troABCD*. This resulted in increased Mn contents and H₂O₂ tolerance.

Oxidative stress tolerance is critical for the ability of bacteria to invade a host. There are multiple mechanisms used by bacteria to resist oxidative stress. In this study, *S. suis* was found to increase the intracellular manganese content in the presence of H₂O₂, and supplementation with manganese can increase the tolerance to H₂O₂ of *S. suis*. Moreover, manganese played an important role in the tolerance to H₂O₂ of *S. suis*. PerR, a Fur family regulator, has been identified in various bacteria and is involved in oxidative stress tolerance. In this study, PerR was found to regulate the sensitivity of bacteria to H₂O₂ by repressing *troABCD* expression. These findings are in accordance with those of a previous report (18). Therefore, manganese plays an important role in oxidative stress defense, and PerR increases the tolerance of *S. suis* to oxidative stress by regulating *troABCD*.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. The bacterial strains, plasmids, and primers used in this study are listed in Tables S1 and S2 in the supplemental material. The *S. suis* strain was cultured at 37°C in brain heart infusion (BHI) broth (Oxoid), in tryptic soy broth (TSB; BD), or on tryptic soy agar (TSA; BD) containing 10% (vol/vol) newborn bovine serum. *Escherichia coli* DH5 α competent cells were grown in Luria-Bertani (LB) broth or on LB agar at 37°C.

Oxidative stress sensitivity assays. The strains were cultured with or without H₂O₂ for 3 h in BHI broth. The bacterial solution was serially diluted 10-fold up to an appropriate dilution for viability counts. The survival rate was calculated by the number of viable strains.

RNA-seq analysis. The *S. suis* wild-type strain was cultured with or without supplementation of 1 mM MnCl₂ until the mid-log phase. The cells were then centrifuged and subjected to RNA extraction. RNA sequencing was performed by Wuhan GeneCreate Biological Engineering Co., Ltd., using an Illumina Novaseq platform. The clean reads were obtained by removing reads containing an adapter, reads containing an N base, and low-quality reads from the raw data. The clean reads were mapped to the *S. suis* SC84 genome using Bowtie2-2.2.3. FPKM, the expected number of fragments per kilobase of transcript sequence per million base pairs sequenced, was used to quantify the levels of gene expression. Differential expression analysis of two groups was performed using the DESeq R package (1.18.0). Genes with an adjusted *P* value of <0.05 obtained by DESeq were considered to be differentially expressed.

Construction of deletion mutants and complementation strains. The method for obtaining a mutant was performed as previously described (45). To obtain a deletion mutant, the up-F/up-R and down-F/down-R primers were used to separately amplify the upstream and downstream regions of the target gene by PCR. The PCR products were directly cloned to a pSET4s vector following digestion with the corresponding restriction enzymes. The recombinant plasmid pSET4s::perR was transformed into SC19. The mutant strain was selected on TSA based on its sensitivity to spectinomycin. The resultant mutant strain was confirmed by PCR and sequencing. The complementary strain was acquired by transforming pSET2, which contained the target gene, into the mutant strain.

RNA extraction and reverse transcription-quantitative real-time PCR. The total RNA in the strains was extracted using a bacterial total RNA isolation kit (Sangon Biotech, China). HiScript II Q RT SuperMix

for reverse transcription and quantitative real-time PCR (qRT-PCR) (+gDNA wiper) (Vazyme, China) was used to synthesize the cDNA. AceQ quantitative PCR (qPCR) SYBR green master mix (Vazyme, China) was used to measure the levels of mRNA. The 16S rRNA gene was used as an endogenous control. The $2^{-\Delta CT}$ method was used to quantify and compare the levels of gene transcription.

H₂O₂ survival assay. Bacterial strains were cultured to mid-log phase. Cells (1 mL) were harvested by centrifugation and subsequently resuspended in phosphate-buffered saline (PBS). The bacterial solution was serially diluted 10-fold up to a 10^{-5} dilution, and 5 μ L of each dilution was spotted onto TSA plates supplemented with 10% (vol/vol) newborn bovine serum and H₂O₂ (0, 5, or 10 mM) or MnCl₂ (1, 0.25, or 0.5 mM). Plates were incubated at 37°C for 12 h.

Analysis of the intracellular manganese content. Measurement of the intracellular manganese content was performed as previously described, with some modifications (46, 47). The harvested strains were washed three times with PBS containing 0.25 mM EDTA and subsequently washed three times with PBS. Cells were then desiccated at 60°C overnight. The dry cell weight was measured, and the cells were resuspended in 35% (vol/vol) HNO₃ and boiled at 95°C for 1 h prior to the removal of debris by centrifugation. Samples were diluted to a final concentration of 3.5% (vol/vol) HNO₃ and analyzed using a flame atomic absorption spectrophotometer.

EMSA. The troA-F and troA-R primers were used to obtain the DNA probe, which was end labeled with biotinylated ribonucleotides. PerR and the DNA probe were incubated in electrophoretic mobility shift assay (EMSA) binding buffer for 20 min, and the mixture was separated on a 4% (wt/vol) native polyacrylamide gel at 70 V and then transferred to a positively charged nylon membrane at 100 V for 20 min, followed by UV cross-linking and incubation with horseradish peroxidase (HRP). Finally, the probe was detected using a ChemiDoc Touch imaging system (Bio-Rad, USA).

Statistical analysis. GraphPad Prism 7 software was used to analyze the data. Student's *t* test for analysis of variance was used to analyze the results. For all tests, a *P* value of <0.05 was considered the threshold for significance.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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We report no potential conflict of interest.

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