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RESEARCH PAPER



A novel aquaporin Aagp contributes to *Streptococcus suis* H₂O₂ efflux and virulence

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ABSTRACT

Streptococcus suis is a bacterium that can cause infections in pigs and humans. Although oxidative stress is common occurrence during bacterial growth and infection, the regulation networks of *S. suis* under oxidative stress remain poorly understood. To address this, we utilized RNA-Seq to reveal the transcriptional landscape of *S. suis* in response to H₂O₂ stress. We identified novel genes responsible for *S. suis* resistance to oxidative stress, including those involved in DNA repair or protection, and essential for the biosynthesis of amino acids and nucleic acids. In addition, we found that a novel aquaporin, Aagp, belonging to atypical aquaglyceroporins and widely distributed in diverse *S. suis* serotypes, plays a crucial role during H₂O₂ stress. By performing oxidative stress assays and measuring the intracellular H₂O₂ concentrations of the wild-type strain and Aagp mutants during H₂O₂ stress, we found that Aagp facilitated H₂O₂ efflux. Additionally, we found that Aagp might be involved in glycerol transport, as shown by the growth inhibition and H₂O₂ production in the presence of glycerol. Mice infection experiments indicated that Aagp contributed to *S. suis* virulence. This study contributes to understanding the mechanism of *S. suis* oxidative stress response, *S. suis* pathogenesis, and the function of aquaporins in prokaryotes.

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Introduction


Streptococcus suis can cause systemic diseases such as septicæmia and meningitis in pigs. It is also considered as a zoonotic pathogen for humans in close contact with infected pigs or contaminated by-products [1–3]. During the growth and infection process, *S. suis* may encounter oxidative stress. Reactive oxygen species (ROS) like hydroxyl radicals (HO•), hydrogen peroxide (H₂O₂), and superoxide anion (O₂^{•−}) are generated during many cellular activities [4]. O₂^{•−} and H₂O₂ result from one-electron and two-electron reduction of O₂, respectively [5,6]. H₂O₂ can oxidize Fe²⁺ in ferritin, leading to inactivation of the enzymes and releasing Fe³⁺, and then Fe³⁺ is reduced to Fe²⁺ in the intracellular environment [5]. When Fe²⁺ reacts with H₂O₂, Fenton chemistry is occurred and generates highly-reactive HO• [7]. O₂^{•−}, H₂O₂, and HO• can cause cell damage by oxidizing amino acids, DNA, and lipids [5,8]. When bacteria invade the host, innate immune cells, including neutrophils, monocytes, and macrophages, are responsible for

ROS production, which is essential to eliminate bacteria [9]. In addition, ROS participates as a signal molecular in activating various immune mechanisms [10]. In order to defend against oxidative stress, *S. suis* has developed various mechanisms that are regulated by transcriptional regulators, including PerR, Rex, SpxA1, FlpS, and SrtR, which target genes encoding superoxide dismutase, catalase, thioredoxin, glutathione reductase, iron uptake (Fur), and ferritin [11–15]. However, since antioxidant defence systems involve comprehensive and complex processes, studies focusing solely on individual regulators cannot fully reveal regulation networks under oxidative stress.

In this study, we performed RNA-sequencing (RNA-Seq) analysis to explore the global oxidative stress response of *S. suis*. Our findings revealed a comprehensive defence network in response to H₂O₂ stress, and we identified a novel aquaporin, Aagp, that plays a crucial role in facilitating *S. suis* H₂O₂ efflux and contributes to virulence.

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Materials and methods

Bacterial strains and culture conditions

Supplementary Table S1 lists *S. suis* strains and plasmids. *S. suis* serotype 9 virulent strain GZ0565 was isolated from a meningitis pig [16]. *S. suis* strains were cultured in Todd-Hewitt broth (THB, Hopebio, Qingdao, China) and plated on an agar (THA) medium containing 6% (vol/vol) sheep blood at 37 °C with 5% CO₂. *Escherichia coli* strains were grown in Luria-Bertani (LB, Becton Dickinson, USA) broth at 37 °C. Antibiotics were added when in need as follows: spectinomycin (Spc, Macklin, China), 50 µg/mL for *E. coli*, and 100 µg/mL for *S. suis*, 10% (w/v) sucrose (sucrose, Macklin, China) for *S. suis*.

For studying glycerol utilization, *S. suis* strains were grown in THB to mid-exponential phase (OD₆₀₀ = 0.6), washed twice in PBS, and then transferred (with a dilution of 1:100) to a 50 mL flask with 10 mL of 1/3 diluted THB or 1/3 diluted THB containing glycerol with a final concentration of 10 mM or 100 mM. The growth rates were measured at OD₆₀₀ at 37 °C in a shaking incubator.

RNA-Seq analysis

To serve as a control group, *S. suis* strain GZ0565 was grown in THB to reach the mid-exponential phase. For H₂O₂ treatment group, when the bacteria had reached the mid-exponential phase, a final concentration of 25 mM H₂O₂ was added to THB medium and incubated for 25 min. After incubation, the cultures were centrifuged at 5000×g for 10 min at 4 °C, and the bacterial pellets were collected. The total RNA was extracted using the FastRNA Pro Blue Kit (MP Biomedicals) according to our previous report [17]. The extracted RNA samples were submitted to Realbio Technology (Shanghai, China) for RNA-seq analysis. Each experimental group was performed in duplicate. The library construction for RNA-seq was described in our previous study [18]. Sequencing was carried out using Illumina HiSeq 2500 (Illumina, USA) following the manufacturer's protocol. The sequencing reads were aligned by Bowtie2 [19]. The gene expression was calculated by FPKM (Fragments Per Kilobase of transcript per Million mapped reads), and differential expression analysis was performed using edgeR [20]. The threshold of $p < 0.01$ and the absolute value of Fold change ≥ 2 were used to identify the differentially expressed genes.

Construction mutant strains

The detailed protocol for deleting *Aagp* (BFP66_RS01250) in strain GZ0565 background using *S. suis*-*E. coli* shuttle

plasmid pSET4s was described in our previous study [21]. In addition, we created a mutation strain of *Aagp* in strain GZ0565 background (*Aagp*^{mut}) by inserting single "G" base into its coding sequence (CDS), causing a frameshift that rendered *Aagp* non-functional. This mutation strain was constructed through a two-step natural transformation [22]. Step I was performed to replace the upstream sequence of *Aagp* with *SacB-Spc* cassette and resulted in sucrose sensitive and spectinomycin resistant; step II was the process of cassette replacement for single base insertion via the negative selection on sucrose THA plate (Supplementary Figure S1). We also employed the same methodology to construct deletions of *exodeoxyribonuclease III* (*exo III*, BFP66_RS05940), *AguB* (BFP66_RS06520), *metQ* (BFP66_RS08225) in strain GZ0565 background, as well as *Aagp* in serotype 2 strain P1/7 background (*Aagp*_{P1/7}). Supplementary Table S2 contains a list of primers used for constructing all these strains.

Oxidative stress assays

To assess the role of the *Aagp* in oxidative stress response, *S. suis* strains were challenged with H₂O₂. Wild-type strain GZ0565 (WT), Δ *Aagp*, and *Aagp*^{mut} were cultured to the mid-exponential phase. Then the final concentration of 25 mM H₂O₂ was added to THB. After incubation at 37 °C for 25 min, the number of bacteria was determined by spreading serial dilutions on THA plates. The survival rate at each time point was calculated as CFU at time point 25 min/CFU at time point 0. The functions of *exo III*, *AguB*, *metQ* and *Aagp*_{P1/7} in response to oxidative stress were evaluated using the same methodology. Experiments were performed with three biological replicates. The statistical analysis was performed with a two-tailed unpaired *t* test.

To further assess the susceptibility of WT, Δ *Aagp*, and *Aagp*^{mut} to H₂O₂, the Oxford cup supplemented with 10 µL 1 M H₂O₂ was conducted on the THA plates. After incubation at 37 °C with 5% CO₂ for 24 h, a transparent circle formed, indicating an inhibition zone.

Measurement of H₂O₂ in cells

After treated with 25 mM H₂O₂ for 25 min, one mL bacterial cultures were centrifuged at 5000 × g at 4 °C for 10 min and subsequently washed once with PBS and resuspended in 1 mL PBS. According to the manufacturer's protocol, the concentration of H₂O₂ in cells was measured using the Pierce Quantitative Peroxide Assay Kits (Thermo Fisher Scientific, Shanghai, China). Experiments were performed with two biological replicates and repeated two times. A two-tailed unpaired *t* test was used for statistical analysis.

Determination of viable bacteria in organs

The virulence of WT and $\Delta Aagp$ was assessed in mice according to our previous report [23]. Mice infection was carried out in the Laboratory Animal Center of Nanjing Agricultural University with the approval of the institution's ethics committee (Permit number SYXK (Su) 2021–0086). Bacteria were cultured to the mid-exponential phase and washed twice with PBS. Six-week SPF CD1 female mice (SiPeiFu Biotechnology Co., Ltd, China) were used for infection (five mice per group). The mice were intraperitoneally injected with a dose of 1.5×10^8 CFU of the WT or $\Delta Aagp$. All mice were euthanized at 24 h post-infection. Blood samples were collected from the heart, and liver, kidney, and brain samples were taken, weighed, suspended in PBS, and homogenized. Plating serial dilutions on THA determined the number of viable bacteria in organs and blood. Experimental results were shown as mean \pm standard error of the mean (SEM). The statistical analysis was performed with a two-tailed unpaired *t* test.

RNA extraction and quantitative real-time PCR (RT-qPCR)

H₂O₂ treatment and RNA extraction were performed as described above for RNA-Seq analysis. The detailed protocol for RT-qPCR was described previously [18]. Supplementary Table 2 lists primers for RT-qPCR analysis. The gene *BFP66_RS5620* (*ParC*) was used as the internal control. Three biological replicates were used.

The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold change. Data were shown as mean \pm SEM.

Results

The transcriptional landscape of *S. suis* oxidative response

The RNA-Seq was used to explore the transcriptional landscape of *S. suis* in response to H₂O₂. Compared with the control condition in the THB medium, 371 differently expressed genes (DEGs) were observed in the H₂O₂ treatment condition, consisting of 112 upregulated and 259 downregulated genes (Supplementary Table 3). The putative functions of these genes were classified into different functional categories according to the gene ontology (GO) (Supplementary Figure S2A). The DEGs are involved in various biological processes, such as biosynthesis of amid acids, carbohydrate transport and metabolism, metal ion transport, and transcriptional regulation.

As shown in Figure 1a, we found that genes involved in DNA repair or protection were upregulated under H₂O₂ stress, including *BFP66_RS05940* encoding an exodeoxyribonuclease III (exo III) and *BFP66_RS06520* encoding an N-carbamoylputrescine amidase (*AguB*). The gene expression of enzymes encoded by *BFP66_RS01580*, *BFP66_RS05715*, *BFP66_RS06935*, *BFP66_RS07745*, *BFP66_RS04160* (*LDH*) was upregulated under H₂O₂ condition, and these enzymes contribute to producing

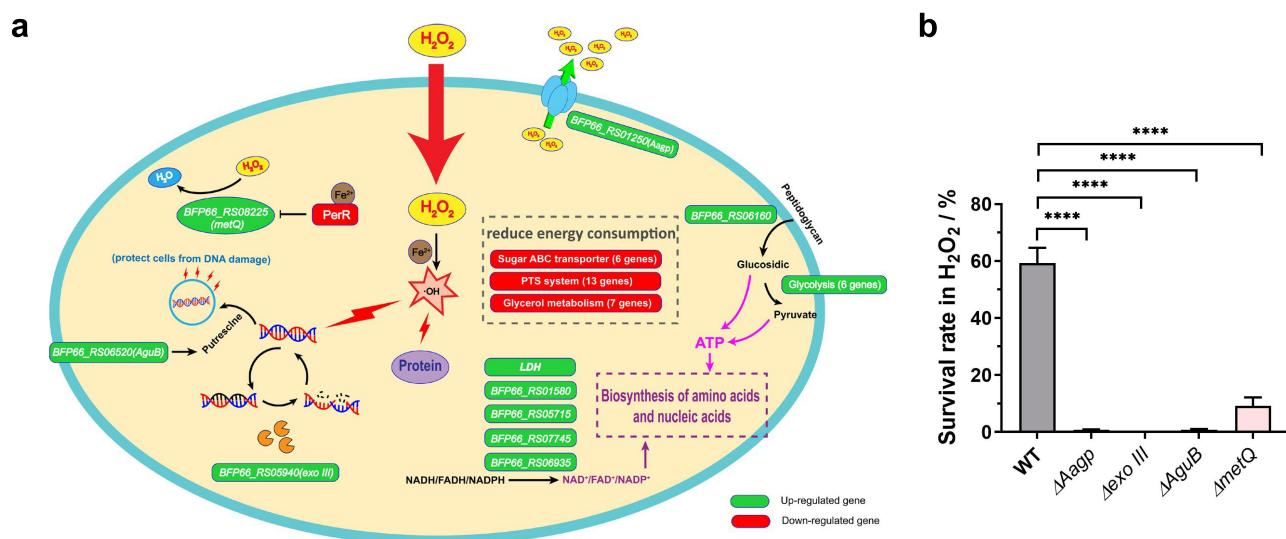


Figure 1. The transcriptional landscape of *S. suis* oxidative response. (a) RNA-Seq analysis was performed to explore the global H₂O₂ stress response of *S. suis*. The upregulated genes are shown in green, and the downregulated genes are shown in red. The detailed information and fold change of genes shown in this figure are summarized in table 1. (b) The survival rate of WT, $\Delta Aagp$, $\Delta exo III$, $\Delta AguB$, and $\Delta metQ$ was determined after 25 min treated with 25 mM H₂O₂. The statistical analyses were performed with a two-tailed unpaired *t* test. “****” indicate *p* < 0.001.

oxidized dinucleotide cofactors (NAD⁺, FAD⁺, and NADP⁺) which are essential for the biosynthesis of amino acids and nucleic acids. As shown in Table 1 and Figure 1, six genes involved in glycolysis were upregulated under H₂O₂ condition, contributing to producing more ATP. In contrast, several genes related to energy-consuming pathways were downregulated, including six genes related to sugar ABC transporters, 13 related to the PTS system, and seven related to glycerol metabolism. RT-qPCR was performed to verify the transcriptional data. Eight upregulated and four downregulated genes were selected, and their expressions were consistent with those obtained from the RNA-seq (Supplementary Figure S2B).

To further investigate the response of *S. suis* to H₂O₂ at the transcriptional level, we selected four DEGs, namely *exo III*, *AguB*, *metQ* (encoding a methionine transporter) and *Aagp* (encoding an aquaporin), to examine their function under oxidative stress conditions. After subjecting the deletion mutations to H₂O₂ treatment, the survival rates of mutants $\Delta Aagp$, $\Delta exo III$, $\Delta AguB$, and $\Delta metQ$ were 0.7075%, 0.0063%, 0.69%, and 9.1867%, respectively, which were significantly lower than that of wild-type (WT) strain (59.3133%) (Figure 1b). These findings suggest that these genes are involved in oxidative stress responses and support the transcriptional landscape data of *S. suis* in response to H₂O₂.

Table 1. The key differentially expressed genes under H₂O₂ stress^a.

ID	Fold Change (H ₂ O ₂ /THB)	Product	Function
<i>BFP66_RS01250</i>	3.66	aquaporin <i>Aagp</i> ;	H ₂ O ₂ efflux
<i>BFP66_RS01545</i>	-3.62	transcriptional regulator <i>PerR</i> ;	oxidative stress regulation
<i>BFP66_RS09925</i>	-1.93	transcriptional regulator <i>mntR</i> ;	oxidative stress regulation
<i>BFP66_RS08225</i>	1.35	methionine ABC transporter, <i>MetQ</i>	methionine transporter, converting H ₂ O ₂ to H ₂ O
<i>BFP66_RS06520</i>	2.01	N-carbamoylputrescine amidase;	converting agmatine to putrescine, which protects DNA from damage
<i>BFP66_RS05940</i>	2.89	exodeoxyribonuclease III;	DNA repair
<i>BFP66_RS06160</i>	2.19	N-acetyl-beta-hexosaminidase;	producing sugar by cell wall hydrolyzation
<i>BFP66_RS04160</i>	2.61	L-lactate dehydrogenase <i>LDH</i> ;	generating NAD ⁺ /FAD ⁺ /NADP ⁺ for biosynthesis of amino acids and nucleic acids
<i>BFP66_RS01580</i>	2.02	NADP-dependent oxidoreductase;	
<i>BFP66_RS05715</i>	2.30	NADH oxidase;	
<i>BFP66_RS07745</i>	2.09	nicotinate phosphoribosyltransferase;	
<i>BFP66_RS06935</i>	2.21	flavodoxin;	
<i>BFP66_RS01665</i>	3.07	fructose-bisphosphate aldolase;	glycolysis
<i>BFP66_RS07495</i>	2.61	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase;	
<i>BFP66_RS06470</i>	2.19	pyruvate kinase;	
<i>BFP66_RS06865</i>	2.36	enolase;	
<i>BFP66_RS02590</i>	2.86	triose-phosphate isomerase;	
<i>BFP66_RS00790</i>	2.16	type I glyceraldehyde-3-phosphatedehydrogenase;	
<i>BFP66_RS00845</i>	-2.35	sugar ABC transporter substrate-binding protein;	sugar ABC transporters
<i>BFP66_RS00850</i>	-2.22	sugar ABC transporter permease;	
<i>BFP66_RS02995</i>	-8.65	sugar ABC transporter permease;	
<i>BFP66_RS07040</i>	-15.41	sugar ABC transporter permease;	
<i>BFP66_RS07045</i>	-10.83	sugar ABC transporter substrate-binding protein;	
<i>BFP66_RS08960</i>	-2.20	sugar ABC transporter substrate-binding protein;	
<i>BFP66_RS00910</i>	-7.00	PTS sugar transporter subunit IIC;	PTS system
<i>BFP66_RS02210</i>	-3.46	PTS system mannose/fructose/N-acetylgalactosamine-transporter subunit IIB;	
<i>BFP66_RS02215</i>	-1.90	PTS mannose/fructose/sorbose/N-acetylgalactosamine transporter subunit IIC;	
<i>BFP66_RS02695</i>	-6.00	PTS glucose transporter subunit IIBC;	
<i>BFP66_RS03575</i>	-14.07	PTS N-acetylgalactosamine transporter subunit IIA;	
<i>BFP66_RS04315</i>	-3.47	PTS lactose/cellobiose transporter subunit IIA;	
<i>BFP66_RS04320</i>	-3.65	PTS lactose transporter subunits IICB;	
<i>BFP66_RS08510</i>	-10.66	PTS cellobiose transporter subunit IIC;	
<i>BFP66_RS08530</i>	-53.00	PTS cellbiose transporter subunit IIC;	
<i>BFP66_RS08570</i>	-5.96	PTS beta-glucoside transporter subunit EIIBCA;	
<i>BFP66_RS09840</i>	-2.24	PTS sugar transporter subunit IIC;	
<i>BFP66_RS09850</i>	-4.61	PTS lactose/cellobiose transporter subunit IIA;	
<i>BFP66_RS09855</i>	-3.09	PTS sugar transporter subunit IIB;	
<i>BFP66_RS05750</i>	-3.95	aquaporin family protein <i>GlpF</i> ;	glycerol metabolism
<i>BFP66_RS05760</i>	-1.66	glycerol kinase <i>GlpK</i> ;	
<i>BFP66_RS05755</i>	-3.12	type 1 glycerol-3-phosphate oxidase <i>GlpO</i> ;	
<i>BFP66_RS05785</i>	-12.96	glycerol dehydrogenase <i>GldA</i> ;	
<i>BFP66_RS05790</i>	-51.82	acetaldehyde/alcohol dehydrogenase;	
<i>BFP66_RS05800</i>	-2.54	Formate C-acetyltransferase/glycerol dehydratase family glycol radical enzyme;	
<i>BFP66_RS09160</i>	-2.28	dihydroxyacetone kinase transcriptional activator <i>DhaS</i> ;	

Note: ^a The threshold of $p < 0.01$ was used to identify the differentially expressed genes.

Aagp, a novel aquaporin, is widely distributed in *S. suis*

Out of the four genes associated with oxidative stress responses, *Aagp* showed the highest fold change (Table 1), making it the chosen candidate for further investigation. Aquaporins contain an aromatic/arginine (ar/R) substrate selectivity motif that comprises one arginine and three other amino acids, and these three amino acids are conserved in every aquaporin subfamily [24]. In *S. pneumoniae*, a new aquaporin subfamily called atypical aquaglyceroporin has YVPR as the substrate selectivity motif, which is different from glycerol-transporting aquaporins with WG(F/Y)R as the motif and water-transporting aquaporins with F(H/I)XR as the motif (Figure 2). *S. suis* aquaporin *Aagp* shares 68.17% amino acid identity with *S. pneumoniae* atypical aquaglyceroporin Pn-AqpC [25], belonging to a novel aquaporin subfamily. *Aagp* was predicted to be a transmembrane protein with five transmembrane loops, and it possesses substrate-selective residues YVPR as Pn-AqpC (Figure 2).

A search for *Aagp* in the NCBI database revealed that 126 *S. suis* strains contained *Aagp*, distributed across numerous serotypes, including serotypes 1–9, 12, 16, 19, 24, 28, and 31 (Figure 3a). To confirm the function of *Aagp* further, a deletion strain of *Aagp*_{P1/7} was constructed in *S. suis* serotype 2 strain

P1/7. When challenged with H₂O₂, the survival rate of Δ *Aagp*_{P1/7} was reduced to 15.1%, compared to 37.0775% for strain P1/7 (Figure 3b). These findings provide further evidence supporting the role of *S. suis* *Aagp* in oxidative stress responses.

Aagp facilitates H₂O₂ efflux and alleviates oxidative stress

Tong *et al.* reported that an aquaporin, So-AqpA, of *Streptococcus oligofermentans* contributes to the diffusion of H₂O₂ across the membrane [26]. To investigate the involvement of *Aagp* in H₂O₂ diffusion, we measured the concentration of H₂O₂ in bacterial cells treated with 25 mM H₂O₂. The concentration of H₂O₂ treated for 25 min in WT (59.22% survival rate) was 8.4067 pmol per 10⁶ cells, which was dramatically lower than 430.731 pmol per 10⁶ cells in Δ *Aagp* (0.96% survival rate) (Figure 4a,b). Despite several attempts, we failed to construct an *Aagp* complementary vector based on plasmids pSET1, pSET2, and pSET3. Therefore, we created a frameshift mutation strain, *Aagp*^{mut}, by inserting one base into its CDS, as we reported in a previous study [27]. Similar to the results obtained from Δ *Aagp*, we found that the survival rate was lower in *Aagp*^{mut} compared with WT, while the concentration of H₂O₂ in *Aagp*^{mut} was significantly

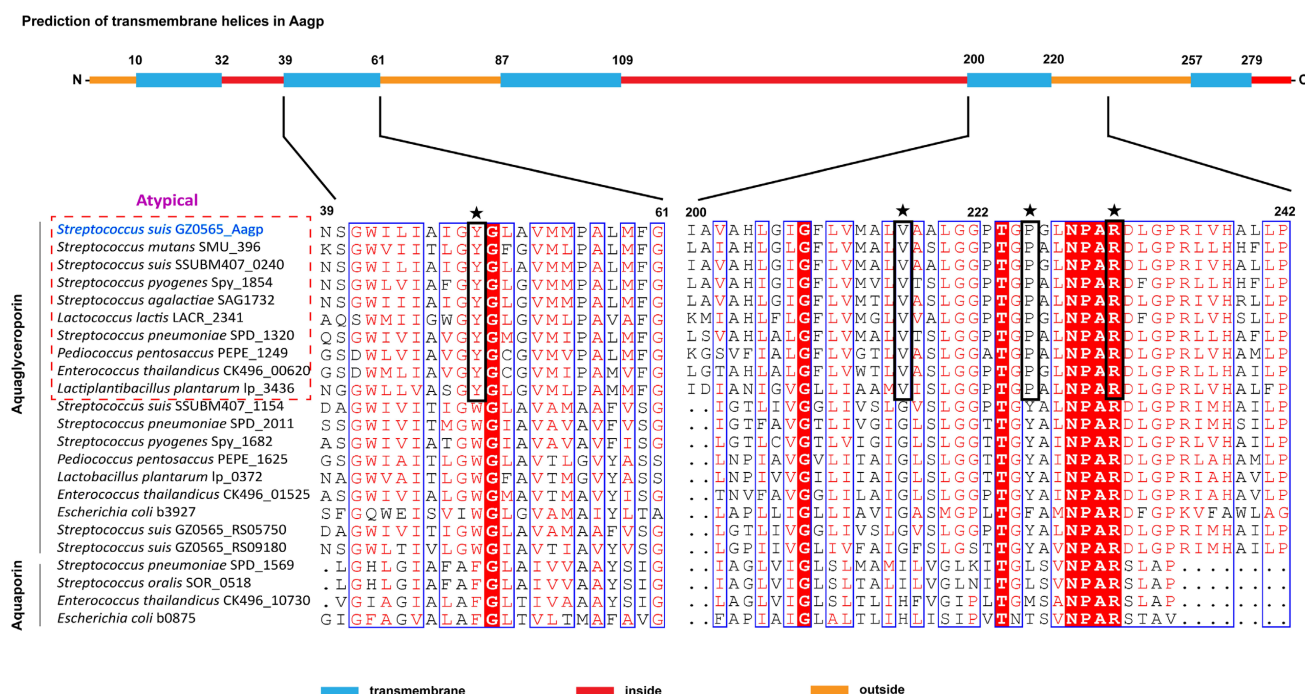


Figure 2. *S. suis* Aagp belongs to atypical aquaglyceroporins. The *S. suis* Aagp transmembrane helices were predicted (top panel). Asterisks specify the ar/R region residues, and black boxes mean the atypical aquaglyceroporins' substrate-selective residues (YVPR).

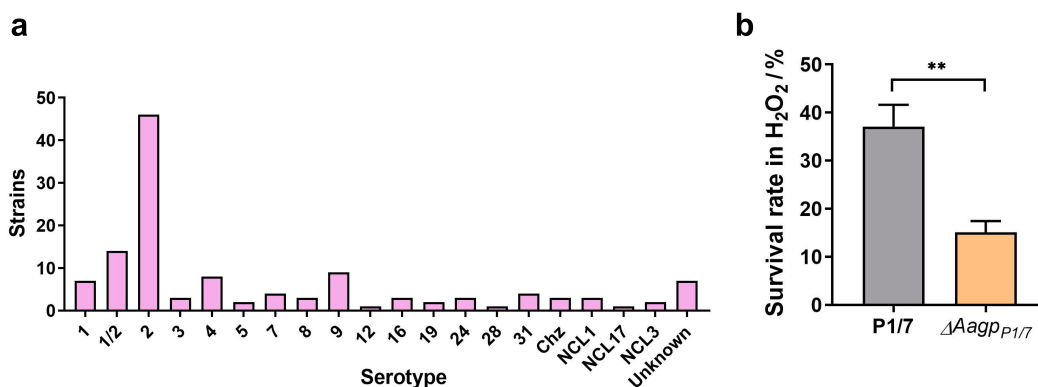


Figure 3. Aagp, a novel aquaporin, is widely distributed in *S. suis*. (a) The distribution of Aagp was assessed in 126 *S. suis* strains by examining Aagp sequences obtained from the NCBI database. (b) The survival rates of P1/7 and $\Delta Aagp_{P1/7}$ were determined after 25 min treated with 25 mM H_2O_2 . The statistical analyses were performed with a two-tailed unpaired *t* test. “**” indicate $p < 0.01$.

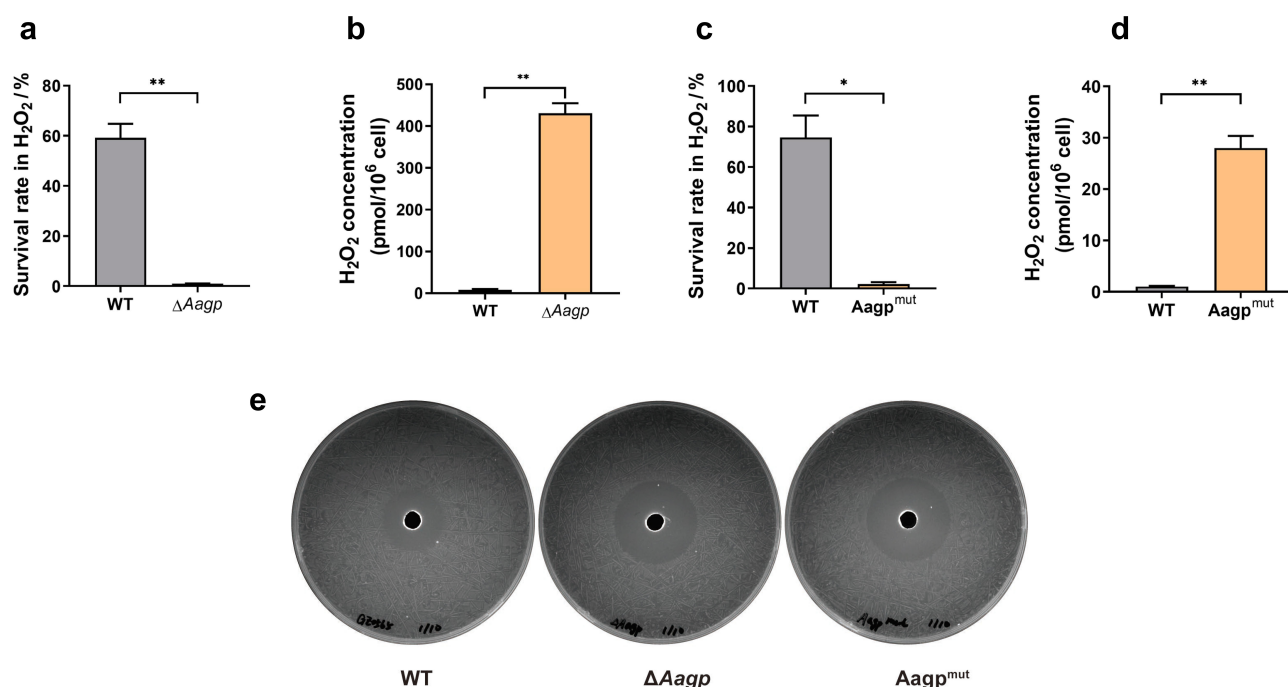


Figure 4. Aagp facilitates H_2O_2 efflux. (a) The survival rates of WT and $\Delta Aagp$ were determined after 25 min treated with 25 mM H_2O_2 . (b) The H_2O_2 concentrations of WT and $\Delta Aagp$ were measured after 25 min treated with 25 mM H_2O_2 . (c) The survival rates of WT and $Aagp^{mut}$ were determined after 25 min treated with 25 mM H_2O_2 . (d) The H_2O_2 concentrations of WT and $Aagp^{mut}$ were measured after 25 min treated with 25 mM H_2O_2 . (e) The response to H_2O_2 between the wild type and Aagp mutations via the Oxford cup test. The statistical analyses were performed with a two-tailed unpaired *t* test. “*” indicates $p < 0.05$ and “***” indicates $p < 0.01$.

higher than in WT (Figure 4c,d). Additionally, we compared the response to H_2O_2 between the WT and *Aagp* mutations via the Oxford cup test. We found that both $\Delta Aagp$ and $Aagp^{mut}$ strains exhibited larger inhibition zones than the WT strain (Figure 4e). These results collectively indicate that Aagp facilitates H_2O_2 efflux, thereby alleviating oxidative stress.

Aagp may be involved in glycerol transport

Previous studies have shown that some aquaporins facilitate glycerol diffusion [28]. To determine the function of Aagp in relation to glycerol, the growth curves of $\Delta Aagp$ and WT were performed in diluted THB medium (1/3 THB as a poor medium condition) containing 10 mM glycerol or 100 mM glycerol under

aerobic condition. As shown in Figure 5a, b, the growth of WT was inhibited as the concentration of glycerol increased, whereas the growth of $\Delta Aagp$ was not affected in the absence or presence of glycerol. As shown in Figure 5c, *S. suis* strain GZ0565 possesses genes involved in the phosphorylation pathway for glycerol metabolism, which is an energy-consuming pathway. Under the aerobic condition, glycerol may be phosphorylated by glycerol kinase (GlpK) and then oxidized by glycerol-3-phosphate oxidase (GlpO), which is accompanied by H_2O_2 production that can be toxic to bacteria [29]. To test this hypothesis, the concentrations of H_2O_2 in WT and $\Delta Aagp$ were measured in 1/3 THB containing 10 mM glycerol or 100 mM glycerol. The concentration of H_2O_2 in WT cultured in 1/3 THB was 0.0178 pmol per 10^6 cells, but in the presence of additional glycerol, the concentration of H_2O_2 increased and reached to 0.1007 pmol per 10^6 cells when cultured in 1/3 THB containing 100 mM glycerol. Conversely, in $\Delta Aagp$, the concentration of H_2O_2 was similar in the absence or presence of glycerol (Figure 5d). These results suggest that Aagp may be involved in glycerol transport.

Aagp contributes to *S. suis* virulence in a mouse infection model

Given that Aagp alleviates *S. suis* oxidative stress, the contribution of Aagp to *S. suis* virulence was assessed in mice. Compared with WT infection groups, the number of $\Delta Aagp$ in blood, liver, spleen, kidney, and brain tissues was significantly reduced, indicating that Aagp contributes to *S. suis* virulence (Figure 6). These results indicate that Aagp contributes to *S. suis* virulence in a mouse infection model.

Discussion

Oxidative stress is a common challenge for *S. suis* to overcome in response to infection or environmental conditions. In this study, we employed RNA-seq to investigate the global transcriptional landscape of *S. suis* under H_2O_2 treatment and to reveal the adaptation mechanisms of *S. suis* response to oxidative stress. Our findings showed that multiple genes involved in oxidative stress were differentially expressed. Some of them have been identified in previous studies. We

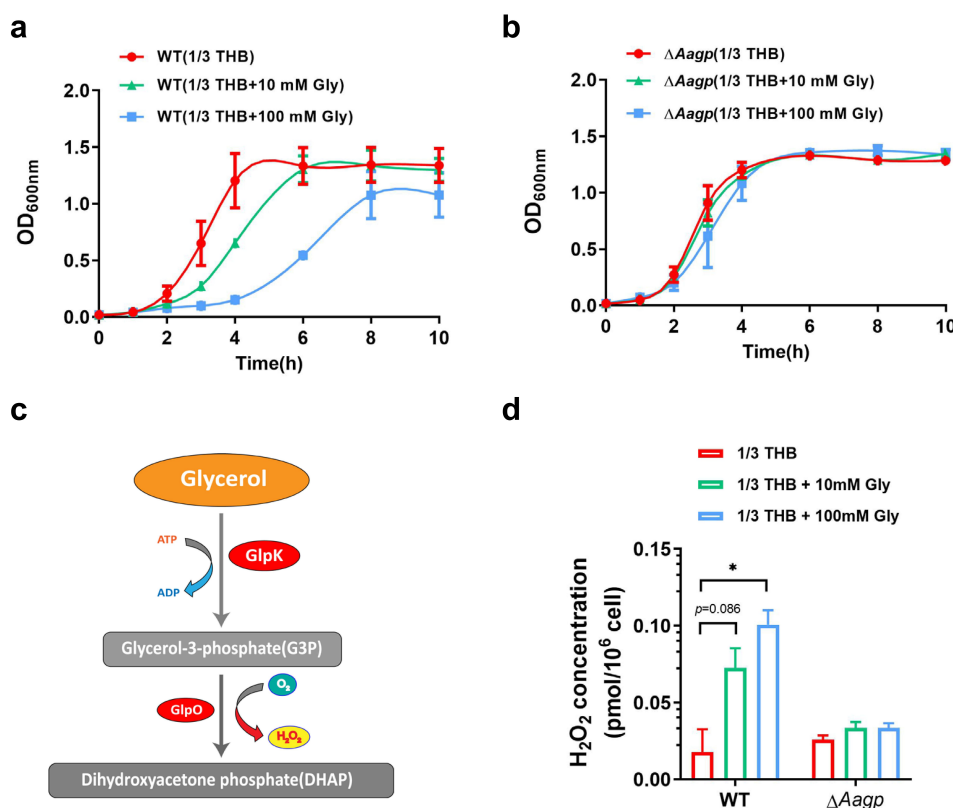


Figure 5. Aagp is involved in glycerol transport. The growth curves of WT (a) and $\Delta Aagp$ (b) cultured in 1/3 THB, 1/3 THB containing 10 mM glycerol or 100 mM glycerol were shown. (c) *S. suis* strain GZ0565 possesses a phosphorylation pathway for glycerol metabolism. The downregulated genes are shown in red in H_2O_2 treatment compared with THB condition by RNA-Seq analysis. (d) The H_2O_2 concentrations of WT and $\Delta Aagp$ cultured in 1/3 THB, 1/3 THB containing 10 mM glycerol or 100 mM glycerol were determined. The statistical analyses were performed with a two-tailed unpaired *t* test. “*” indicates $p < 0.05$.

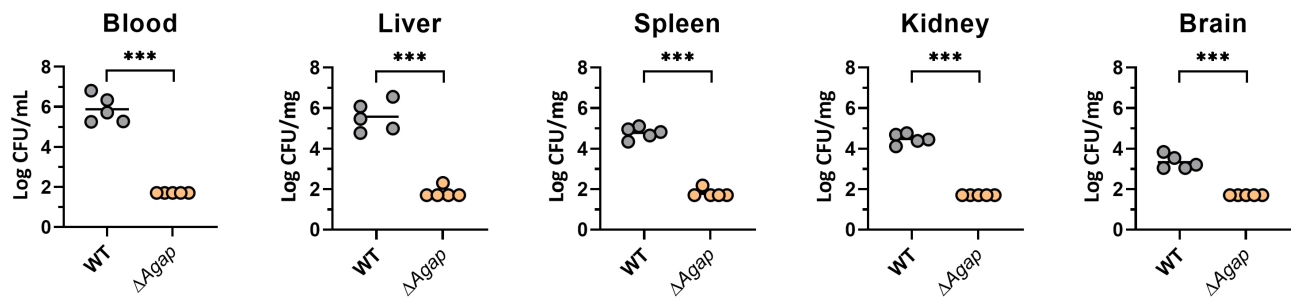


Figure 6. Aagp contributes to *S. suis* virulence in a mouse infection model. Five mice per group were injected intraperitoneally with 1.5×10^8 CFU of WT and $\Delta Aagp$. All mice were euthanized at 24 h post-infection. Bacteria from blood, liver, spleen, kidney and brains were plated onto THA, and colonies were expressed as Log₁₀ CFU/mg or Log₁₀ CFU/mL. The statistical analyses were performed with a two-tailed unpaired *t* test. “***” indicate *p* < 0.001.

found that *BFP66_RS05715* encoding NADH oxidase, known to contribute to oxidative stress tolerance [30], was upregulated 2.30-fold by H₂O₂ treatment. We also observed that *BFP66_RS01615* encoding trigger factor, which contributes to stress tolerance [31], was upregulated 3.12-fold by H₂O₂ treatment. Additionally, we noted a downregulation of *BFP66_RS01545* encoding Fur-like protein PerR by 3.62-fold in response to H₂O₂ stress, consistent with previous research showing that the *PerR* deletion mutant is less sensitive to H₂O₂ stress [12]. *PerR* negatively regulates *metQ*, and its derepression increases methionine utilization, converting H₂O₂ to H₂O [12]. Although RNA-Seq analysis indicated a modest 1.35-fold up-regulation of *metQ* (*BFP66_RS08225*) (*p* < 0.01), RT-qPCR analysis revealed a 10.65-fold upregulation (Supplementary Figure S2B). Moreover, compared to the WT, $\Delta metQ$ was more sensitive to H₂O₂, confirming the role of *metQ* under H₂O₂ conditions (Figure 1b). These findings indicate that the identified genes are crucial in *S. suis* response to oxidative stress.

Our study has uncovered novel mechanisms that may be responsible for *S. suis* resistance against oxidative stress caused by ROS. ROS can cause damage to DNA, proteins, and lipids, and repairing these molecules is crucial to combating oxidative stress. In *E. coli*, exonuclease III is a major apurinic/aprimidinic endonuclease that recognizes base lesions and cleaves the glycosylase bond to repair DNA [32]. We found that the gene *BFP66_RS05940*, which codes for exodeoxyribonuclease III was upregulated in response to H₂O₂ stress, indicating that it may be involved in DNA repair through cleaving lesions. Additionally, *BFP66_RS06520* encoding an N-carbamoylputrescine amidase (*AguB*) was upregulated upon H₂O₂ stress. The *AguAB* pathway converts agmatine to putrescine, protecting *E. coli* from DNA damage via ROS scavenging [33]. Our deletion studies showed that *exo III* and *AguB* are crucial

for H₂O₂ resistance in *S. suis* (Figure 1b). Furthermore, we observed upregulated genes contributing to the biosynthesis of amino acids and nucleic acid or transport, including five genes involved in producing oxidized dinucleotide cofactors (Figure 1a), *BFP66_RS01165* encoding a component of an amino acid ABC transporter, and *BFP66_RS04785* encoding a glutamate dehydrogenase. These findings indicated that *S. suis* enhances its biosynthesis and transport of amino acids and nucleic acid to respond to H₂O₂ stress. Interestingly, under ROS stress caused by 2,4-dichlorophenoxyacetic acid, *E. coli* reduces energy-consuming pathways to conserve energy for vital metabolism [33]. In our study, several genes related to energy-consuming pathways were downregulated under H₂O₂ stress (Figure 1a). In contrast, genes involved in glycolysis were upregulated, indicating that *S. suis* generates more ATP through enhanced glycolysis and reduces energy-consuming pathways for H₂O₂ detoxification.

The aquaporins are divided into three subfamilies: supergene channel superaquaporins, water-transporting aquaporin, and glycerol-transporting aquaglyceroporins [34–36]. Recent studies have demonstrated that aquaporins can transport H₂O₂ and regulate its levels in animals and plants, preventing cells from oxidative stress [37–40]. However, little is known about the function of aquaporins in H₂O₂ regulation in bacteria. *S. oligofermentans* aquaporin So-AqpA, sharing only 21.90% amino acid identity with *S. suis* Aagp, has been reported to facilitate the efflux of endogenous H₂O₂ and protect the bacterium from oxidant damage [26]. Both *S. pneumoniae* Pn-AqpC and *S. suis* Aagp are atypical aquaglyceroporins and contribute to bacterial resistance to H₂O₂ stress, but their mechanisms are different. Pn-AqpC can facilitate oxygen permeation into pneumococcal, which promotes the generation of endogenous H₂O₂ and helps *S. pneumoniae* adapt to higher exogenous

H₂O₂ [25]. In contrast, *S. suis* Aagp contributes to resistance to oxidative stress by facilitating H₂O₂ efflux, similar to So-AqpA. Previous studies have shown that in *S. oligofermentans*, the expression of So-aqpA is negatively regulated by MntR and PerR [26,41]. In our RNA-seq data, we observed a significant reduction in the expressions of *MntR* (encoded by *BFP66_RS09925*) and *PerR* (encoded by *BFP66_RS01545*), accompanied by a significant upregulation of *Aagp* during H₂O₂ stress in *S. suis*. These findings suggest that MntR and PerR in *S. suis* may play similar roles in regulating Aagp under H₂O₂ stress conditions. Additionally, we propose that Aagp may possess dual functions of both H₂O₂ efflux and glycerol transport. In *Lactobacillus plantarum*, GlpF2, GlpF3, and GlpF4, sharing 30.37%, 36.63%, and 32.73% amino acid identity with Aagp, respectively, have been shown to be permeable to both H₂O₂ and glycerol [42]. In our study, we have confirmed that Aagp facilitates H₂O₂ efflux by oxidative stress assays and measurement of intracellular H₂O₂ level. However, further investigation is required to determine the glycerol transport ability of Aagp.

Through in-depth analysis, we have identified homologs of Aagp that play a role in virulence in other pathogenic bacteria. In Group B *Streptococcus*, GlpF, sharing 81% amino acid identity with Aagp, enhances the invasion capacity of Group B *Streptococcus* into eukaryotic cells [43]. In *S. pneumoniae*, Pn-AqpC contributes to pneumococcal pathogenicity by modulating H₂O₂ production and pneumolysin release [25]. In *L. monocytogenes*, the expression of GlpF, sharing 37% amino acid identity with Aagp, is upregulated during intracellular growth [44]. When bacteria invade, host innate immune cells generate ROS such as H₂O₂. Neutrophils, monocytes, and macrophages, abundant in blood and tissues, play crucial roles in producing ROS to eliminate bacteria. The deletion of *Aagp* would impair the defence capability of *S. suis* survival in blood and tissues. Finally, we assume that Aagp contributes to *S. suis* oxidative stress resistance and promotes its survival in the host by facilitating H₂O₂ efflux.

In summary, we depicted *S. suis* defence network response to H₂O₂ stress and identified a novel aquaporin, Aagp, that alleviated *S. suis* oxidative stress via facilitating H₂O₂ efflux, which contributes to virulence. In addition, Aagp might be involved in glycerol transport.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data Availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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