

RESEARCH PAPER



## Analysis of multidrug resistance in *Streptococcus suis* ATCC 700794 under tylosin stress

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### ABSTRACT

*Streptococcus suis* is an important zoonotic pathogen. The massive use of tylosin and other antibiotics in swine production has led to the emergence of resistant phenotypes of *S. suis*. However, there are no adequate measures available to address the problem of bacterial resistance. This study involved the use of 1/4 MIC (0.125 µg/mL) of tylosin to investigate resistance-related proteins by *S. suis* ATCC 700794. Our results showed that 171 proteins were differentially expressed in *S. suis* tested with 1/4 MIC (0.125 µg/mL) of tylosin using iTRAQ-based quantitative proteomic methods. TCS, heat shock protein and elongation factors were differentially expressed at 1/4 MIC (0.125 µg/mL) of tylosin compared to non treated, control cells. Using quantitative RT-PCR analysis, we verified the relationship between the differentially expressed proteins in *S. suis* with different MIC values. The data showed that expression profile for elongation factor G (*fusA*), elongation factor Ts (*tsf*), elongation factor Tu (*tuf*), putative histidine kinase of the competence regulon, ComD (*comD*), putative competence-damage inducible protein (*cinA*) and protein GrpE (*grpE*), observed in tylosin-resistant *S. suis*, correlated with that of *S. suis* ATCC 700794 at 1/4 MIC (0.125 µg/mL). The MIC of tylosin-resistant showed high-level resistance in terramycin, chlortetracycline, ofloxacin and enrofloxacin. Our findings demonstrated the importance of elongation factors, TCS and heat shock protein during development of tylosin resistance in *S. suis*. Thus, our study will provide insight into new drug targets and help reduce bacterial multidrug resistance through development of corresponding inhibitors.

### ARTICLE HISTORY

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### KEYWORDS

*Streptococcus suis*; tylosin; iTRAQ; multidrug resistance

## Introduction


Tylosin is one of the most common macrolide antibiotics, which is effective against both Gram-positive and Gram-negative bacteria [1]. It is widely used to prevent respiratory diseases caused by organisms, such as *Mycoplasma*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus suis* [2]. *S. suis* is an important zoonotic pathogen that causes a wide range of diseases in pigs, including meningitis, septicemia, pneumonia, endocarditis, and arthritis [3]. With the massive use of tylosin, different parts of the world have reported widespread clinical resistance [4,5]. During 2010 to 2013, characterization of 227 *S. suis* strains isolated from pigs showed high levels of resistance to tylosin (93.8 %) in Korea [6]. Moreover, 95.6% of the isolates were resistant to clindamycin, tilmicosin, tylosin, oxytetracycline and more classes of antimicrobials, indicating the high prevalence of multidrug resistant *S. suis*.

Generally, there are at least four classical antibiotic resistance mechanisms including inactivation of drugs

via hydrolysis or modification, alteration or bypass of the drug target, permeability changes in the bacterial cell wall which restricts antimicrobial access to target sites, and active efflux of the antibiotic from the microbial cell. In addition to the above mechanisms, new gene acquisition, gene expression modifications, two-component regulatory systems (TCS), heat shock proteins, and bacterial elongation factor are involved in drug resistance [7,8].

TCS, the most widespread regulatory systems in bacteria [9], are recognized by two-main protagonists: the histidine kinase and their cognate response regulator [10]. The competence operon comCDE and putative competence-damage inducible protein (*cinA*) which are involved in the regulation of TCS, provided evidence for gene transfer events in *Streptococcus pneumoniae* [11]. Meanwhile, previous studies have shown that TCS regulates antibiotic resistance in many pathogenic bacteria [12–15]. Therefore, TCS may play an important role in multidrug resistance. It is well

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 Supplementary data for this article can be accessed [here](#).

known that elongation factors play important roles in forming bonds between the tRNA attached peptidyl-transferase and the next amino acid, ribosomal translation, causing premature dissociation of the peptidyl-tRNA from the ribosome and protein synthesis [16–19]. Previous studies have shown that elongation factors can not only inhibit bacterial protein synthesis using the antibiotics tetracycline, tylosin and fluoroquinolone [20,21], but also take part in imparting drug resistance in *Escherichia coli*. Moreover, elongation factors have been reported to be differentially expressed in tylosin-resistant *Mycoplasma gallisepticum* [22–24]. Thus, elongation factors are considered as potential targets for multi-drug resistant bacteria. In addition, heat shock proteins are involved in antibiotic resistance mechanism. Heat shock proteins are stress-responsive proteins which act as chaperonins to fold proteins. Many heat shock proteins (HSP) such as DnaJ, DnaK, GroEL, GroES, and GrpE have been differentially expressed in drug-resistant strains [25–28]. They play a role in the development of drug resistance by providing coping mechanism during the early stages of exposure to drugs in *E. coli* [29].

Quantitative proteomics have considerably improved during the past decade and been employed in comparative analysis to study whole proteome-wide expression dynamics of cells grown under a variety of growth or stress conditions such as antibiotics [30]. Researchers have shown that quantitative proteomic analysis can be used as an effective tool to find novel resistance mechanisms [31]. However, changes in the proteomics profile of *S. suis* in response to tylosin have not been yet reported.

In our study, iTRAQ labeling-based quantitative proteomics was utilized to study the differentially expressed proteins in *S. suis* upon addition of tylosin in comparison to non-treated, control cells. Bioinformatics analysis revealed that proteins related to TCS, protein elongation factor and heat shock protein could be involved in tylosin resistance mechanism. In order to identify whether these proteins contributed to tylosin-resistance, *S. suis* was induced in the presence of sub-minimum inhibitory concentration (MIC; 0.125 µg/mL) of tylosin, *in vitro*. In the tylosin-resistant strain, candidate proteins were further verified for changes at mRNA level by qRT-PCR. The MICs of tetracycline and fluoroquinolone were determined to confirm resistance to these drugs in the strain and to establish the possibility of multidrug resistance in the presence of tylosin. Our findings demonstrated the importance of TCS, protein elongation factor and HSP in tylosin resistance of *S. suis*, thus providing insight into developing corresponding inhibitors to reduce bacterial resistance.

## Materials and methods

### Bacterial strain and cultivation

*Streptococcus suis* ATCC 700794 was purchased from American type culture collection. *S. suis* was grown in Todd-Hewitt broth (THB) (THB: Summus Ltd, Harbin, Heilongjiang, China) or Todd-Hewitt broth agar (THA) with 5% (*v/v*) fetal bovine serum (Sijiqing Ltd, Hangzhou, Zhejiang, China). The cultures were used for inducing the resistance ATCC 700794 (S-t-R 128) and the MIC assays.

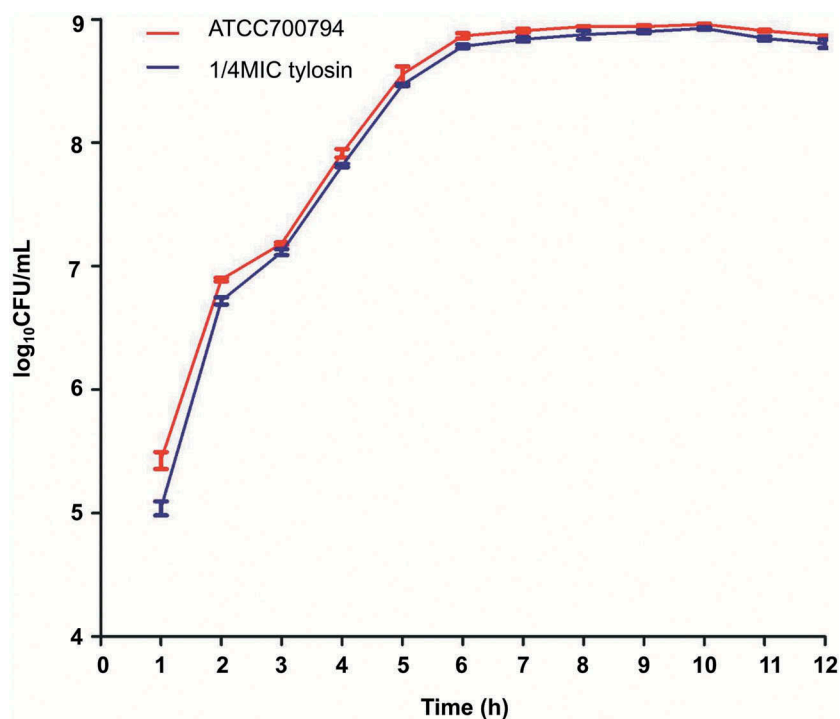
### Minimum inhibitory concentrations and growth rates

The MIC of *S. suis* to tylosin (98% purity, Solaibao Biotechnology Co., Ltd, Beijing, China) was determined by microtitre broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI). Cultures were diluted to  $1 \times 10^6$  colony-forming units (CFU)/mL using THB. Finally, 100 µL of cell suspensions were inoculated into the wells of a 96-well plates (Corning Costar® 3599 Corning, NY, USA) containing serial dilutions of tylosin culture medium as previously reported [32,33]. At the same time, negative control was set up as outlined in a previous study [33]. The inoculated microplates were incubated at 37°C for 24 h. The MICs of tylosin-resistant mutants to a variety of antibiotics, terramycin, chlortetracycline, ofloxacin, enrofloxacin, florfenicol and penicillin K (98% purity, Solaibao Biotechnology Co., Ltd, Beijing, China) were measured using the same procedure.

The growth rates of *S. suis* ATCC 700794 treated with and without 1/4 MIC (0.125 µg/mL) tylosin were analyzed [33]. Briefly, *S. suis* ATCC 700794 treated without tylosin and with 1/4 MIC tylosin (0.125 µg/mL) were incubated at 37°C for 12 h. Then, the samples were taken every hour and applied to the blood plate (THA with 5% (*v/v*) fetal bovine serum). All samples were diluted into  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  and  $10^{-8}$  CFU/mL. 0.1 mL bacterial fluid from  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  dilutions were taken and applied to the plate. The assays were repeated 3 times. These plates were cultured at 37°C overnight. The number of colonies grown on the plate were counted on the 2nd day and there were 20 to 300 colonies per plate [34] (Figure 1).

### iTRAQ analysis

Protein was extracted from *S. suis* cells under two different conditions at 37°C for 24 h (cells treated with 1/4 MIC (0.125 µg/mL) of tylosin and non-treated cells) as described previously [33]. The iTRAQ analysis was



**Figure 1.** Growth curve of *Streptococcus suis* ATCC 700794 in the absence of tylosin and in the presence of 1/4 MIC of tylosin. Data are expressed as means  $\pm$  standard deviations.

carried out at Shanghai Applied Protein Technology Co. Ltd (APT, Shanghai, China). Three biological replicates were evaluated to minimize experimental error. According to the FASP procedure and the reagent protocol [33], iTRAQ-labeled peptides were fractionated by strong cation exchange (SCX) chromatography using the AKTA Purifier system. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer coupled to EasynLC (Proxeon Biosystems, Thermo Fisher Scientific).

### Bioinformatics analysis

The sequence data of 171 differentially expressed proteins were retrieved from Uniprot KB. The differentially expressed patterns were illustrated using K-means clustering in conjunction with heat map (Figure 2) [35]. The relationships between up-regulated and down-regulated proteins were analyzed using STRING database (<http://string-db.org/>). The creation of a PPI network based on experimental data was used to predict the interactions.

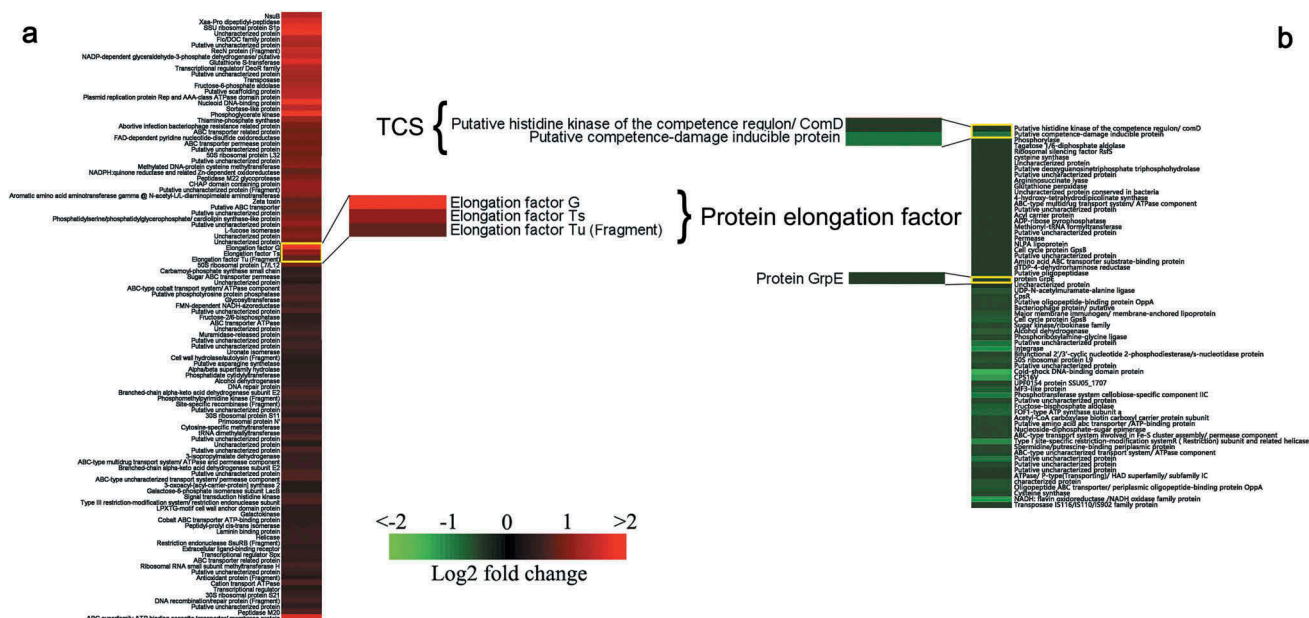
### Selection of tylosin-resistant *S. suis* mutants

*S. suis* was used to select tylosin-resistant mutants [32]. To select resistant mutants, we performed serial passaging in THB medium containing 1/4 MIC (0.125  $\mu$ g/mL) of tylosin. MIC of tylosin was

determined for *S. suis* and resistant strains were induced *in vitro* using the broth dilution method in 96-well plates. In these plates, each well contained diluted concentrations of tylosin with 0.125  $\mu$ g/mL, 0.25  $\mu$ g/mL, 0.5  $\mu$ g/mL, 1  $\mu$ g/mL, 2  $\mu$ g/mL and 4  $\mu$ g/mL. The inoculated microplates were incubated at 37°C for 24 h before evaluated. In addition, the stability of tylosin resistance was tested by serial passage (10 times) on an antibiotic-free medium.

### The quantitative RT-PCR (qRT-PCR)

*S. suis* was grown to mid-log phase and then the culture medium was supplemented with 1/4 MIC (0.125  $\mu$ g/mL) of tylosin prior to further incubation at 37°C for 24 h. Control cells were incubated in the absence of tylosin. Resistant strains at a drug resistance level of 16  $\mu$ g/mL of tylosin (S-t-R 16) and 128  $\mu$ g/mL of tylosin (S-t-R 128) were also incubated at 37°C for 24 h. Total RNA extraction (Omega, Beijing, China) and cDNA synthesis (Takara, Dalian, China) were done according to the manufacturer's instructions. To investigate the effect of tylosin on the expression of genes, six proteins were chosen (Table 1). The 16S rRNA of *S. suis* strain was selected as a reference gene for all the experiments and the primers of target genes are listed in Table 2 [36]. The reaction conditions were 94°C for 10 min followed by 40 cycles of amplification at 94°C for 15 s and 60°C for 60 s [36]. The assays were repeated 3 times.



**Figure 2.** K-means clustering representation of 171 profiles of *Streptococcus suis* with up-regulations (a) and down-regulations (b). The magnitude of the percentage is represented by a color scale (top right) going from low (green) to high (red).

**Statistical analysis**

Each set of values was paralleled with three sets of controls and compared with the control groups. Student’s t-test was used to calculate the differences between the two mean values, with  $p < 0.05$ , set as the statistical significance threshold.

**Results**

**Bacterial growth under the influence of tylosin at sub-MIC**

MIC of tylosin against *S. suis* ATCC 700794 was 0.5 µg/mL in the present study. Furthermore, after 10 h of incubation at 37°C, both non-treated and treated (1/4 MIC (0.125 µg/mL) of tylosin) *S. suis* ATCC 700794 reached stationary phase, indicating there was no effect of tylosin on the growth rates of *S. suis* strain (Figure 1).

**Analysis using iTRAQ**

To investigate the effect of tylosin stress on *S. suis*, 1/4 MIC of tylosin was selected (0.125 µg/mL) for quantitative proteomic analysis. First, bacteria treated and untreated with tylosin were labeled with isobaric reagents. Following this, the samples were pooled, fractionated by SCX chromatography, separated by LC and analyzed by MS/MS. Our analysis detected 1501 proteins. A ratio of proteins with  $> 1.2$  or  $< 0.8$

**Table 1.** Identification of Differentially Expressed Proteins.

Accession	Proteins	Fold change
G75BK2	Elongation factor Ts	2.27
G75MG3	Elongation factor G	3.67
Q9EZW2	Elongation factor Tu	1.74
G5L3D2	Putative histidine kinase of the competence regulon, ComD	0.74
G75LJ0	Putative competence-damage inducible protein	0.56
A4VZB4	Protein GrpE	0.72

**Table 2.** Primers used for the quantitative qRT-PCR analysis.

Genes	Primer sequence
grpE	Forward: 5’AGCCGAGCAGCAACAACAG 3’ Reverse: 5’CTCGCCATCTACCACATCATCTCTG 3’
cinA	Forward: 5’GGTACAGACAGTAGCAGAGCGGATTG 3’ Reverse: 5’AAGTACGTTGCTCTCTGTTGATTCGG 3’
comD	Forward: 5’AGTTCGTTATAGTCGTCATGTTGAGTC 3’ Reverse: 5’ GTTCGAATCGCATCAATGTCTTCTCTC 3’
fus	Forward: 5’CTGGATGGAGCAAGCAAGAGC 3’ Reverse: 5’ACACTGATTGTGAGTCAAGAACGG 3’
tuf	Forward: 5’TTGGTGTGCTTCTCTGTTGTTGATC 3’ Reverse: 5’TACGGAAGTAGAAGTCTGGACGGTAG 3’
tsf	Forward: 5’ AGCAGAATTGGCAGCAGAAGGC 3’ Reverse: 5’ GAAGCGTTCAGTGTCAAGTAAGC 3’
16S rRNA	Forward: 5’ GATATATGGAGGAACCCG 3’ <sup>[36]</sup> Reverse: 5’ GACCAACACCTAGCATC 3’ <sup>[36]</sup>

( $p$ -value $<0.05$ ) was categorized as differentially expressed proteins. A total number of 171 differentially expressed proteins were identified in 1/4 MIC (0.125 µg/mL) of tylosin treated cells when compared to non-treated cells. Due to tylosin stress, 105 proteins were up-regulated, and 66 proteins were down-regulated in *S. suis* treated with tylosin, in comparison to the non-treated sample. In hierarchical clustering

analysis (Figure 2), some proteins were found to have a significant change in their expression as visualized using the R studio software.

The resistance-related proteins like elongation factor G (G7SMG3), elongation factor Ts (G7SBK2), elongation factor Tu (Q9EZW2) were up-regulated and had fold-change values of 3.67, 2.27 and 1.74, respectively. Proteins like putative histidine kinase of the competence regulon, ComD (G5L3D2), putative competence-damage inducible protein (G7SLJ0), protein GrpE (A4VZB4) were down-regulated in tylosin-treated sample with fold-change values of 0.74, 0.56 and 0.72, respectively (Table 1).

### Protein-Protein interaction analysis

Protein-protein interaction and network analysis were performed on 171 differentially expressed proteins using the web-based tool STRING. Among these proteins, 77 proteins were included in the network and connected with each other. These proteins belonged to protein elongation factor, TCS and heat shock protein families (Figure 3). The major networking proteins in the STRING analysis were EF-Tu, Ef-G, EF-Ts, putative competence-damage inducible protein and protein GrpE.

### MIC determination of resistant strain

The tylosin MIC against *S. suis* ATCC 700794 was 0.5 µg/mL. After 25 passages induction, the tested strain could tolerate 128 µg/mL, which MIC increased 256-fold in comparison with the parent strain. The resistance phenotype remained stable after 10 passages in antibiotic-free medium. In CLSI, there is no clear clinical breakpoint for tylosin against *S. suis*, but the strain which could amplify *erm* can represent the resistance to macrolide. In the previous studies, we know the gene of *ermB* can mediate high levels of resistance to tylosin and can be amplified in the resistant strain [37–41]. So, in our study, the *ermB* was used to verify whether the strain was resistant or sensitive to tylosin. The results showed that the strains S-t-R 16, S-t-R 128 could amplify *ermB*, but *S. suis* ATCC 700794 could not amplify *ermB* (Supplementary Material). So, the strains S-t-R 16, S-t-R 128 were tylosin-resistant, while *S. suis* ATCC 700794 was not resistant.

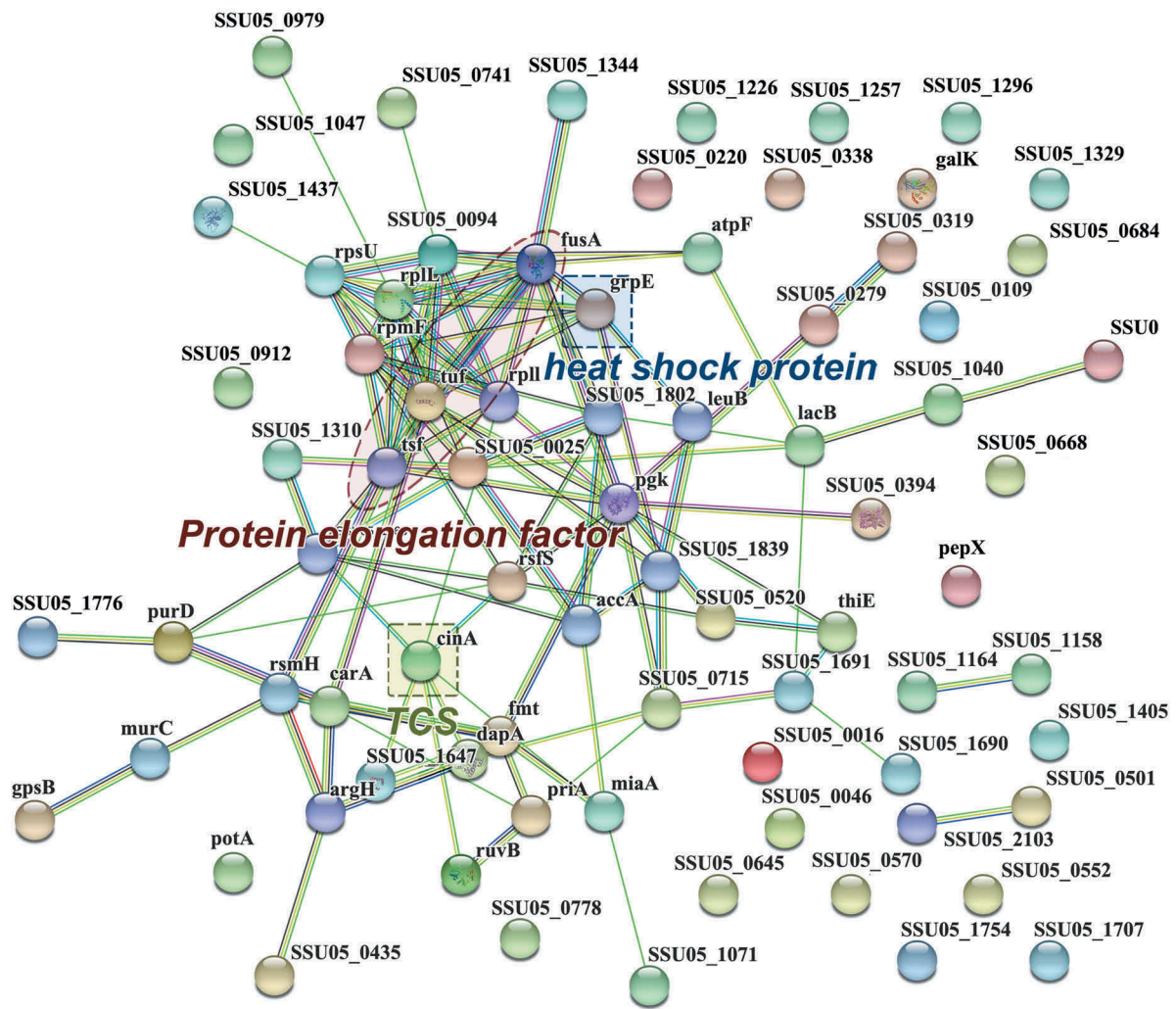
The susceptibility of *S. suis* to different kinds of antibiotics were determined using the *in-vitro* method recommended by the CLSI. The MICs of terramycin, chlortetracycline, ofloxacin and enrofloxacin indicated high-level resistance. However, the MICs of florfenicol and penicillin K were unchanged (Table 3).

### Investigation of changes in mRNA transcript levels in tylosin-resistant *S. suis*

To explore whether the differential expression of proteins reflected a change at the transcription stage, mRNA levels of six proteins in treated with 1/4 MIC (0.125 µg/mL) of tylosin and non-treated *S. suis* were analyzed by qRT-PCR. Transcript levels of elongation factors (*fusA*, *tuf*, *tsf*), GrpE (*grpE*) and TCS (*cinA*, *comD*) were analyzed. Complementing the proteomics data, gene expressions of *grpE*, *cinA* and *comD* were down-regulated at mRNA level, while the gene expression of *fusA*, *tuf* and *tsf* were up-regulated. In order to verify whether the expressions of these proteins related to drug resistance, drug-resistant bacteria was induced and analyzed for changes at the transcription level. In the drug-resistant bacteria, the mRNA levels of *fusA*, *tuf*, *tsf*, *comD* and *grpE* were consistent with that of *S. suis* ATCC 700794 which was treated with 1/4 MIC (0.125 µg/mL) of tylosin (Figure 4(a)). In order to confirm that the change was caused by tylosin, we detected the expression levels of *fusA*, *tsf*, *tuf*, *cinA*, *comD* and *grpE* in *S. suis* ATCC 700794, S-t-R 16 (Figure 4(b)) and S-t-R 128 (Figure 4(c)). With the increase in the level of resistance, the gene expression levels of *fusA*, *tsf* and *tuf* (Figure 4(d)) increased, while that of *cinA*, *comD* and *grpE* decreased (Figure 4(e)).

### Discussion

Generally, antibiotics are the first choice of treatment opted by veterinarians and farmers whenever there is an occurrence of bacterial disease. The long term use or abuse of antibiotics has often resulted in bacterial resistance, as in the cases of bacteria such as *E. coli*, *Staphylococcus aureus* and *Coagulase-Negative Staphylococci* [1]. Thus, an understanding of the mechanisms involved in antibiotic resistance is necessary to extend the life of current antibiotics and enable the discovery of novel targets [6]. The intricate molecular variations within bacterial pathogens associated with antibiotics, in response to treatment bacterial diseases, complicates the identification and quantification of functional proteins. So far, *S. suis* is a major public health issue and an emerging zoonotic agent in Southeast and East Asia [42,43]. In order to find appropriate inhibitors that can reduce bacterial resistance in tylosin-resistant *S. suis*, it is very important to focus on discovering specific target proteins which are related to tylosin resistance or multi-drug resistance. In the last few years, proteomics methods have become a powerful tool for investigating antibiotic resistance mechanisms [44]. In this study, we used an iTRAQ-based proteomic approach to gain a better



#### Known Interactions

- from curated databases
- experimentally determined

#### Predicted Interactions

- gene neighborhood
- gene fusions
- gene co-occurrence

#### Others

- textmining
- co-expression
- protein homology

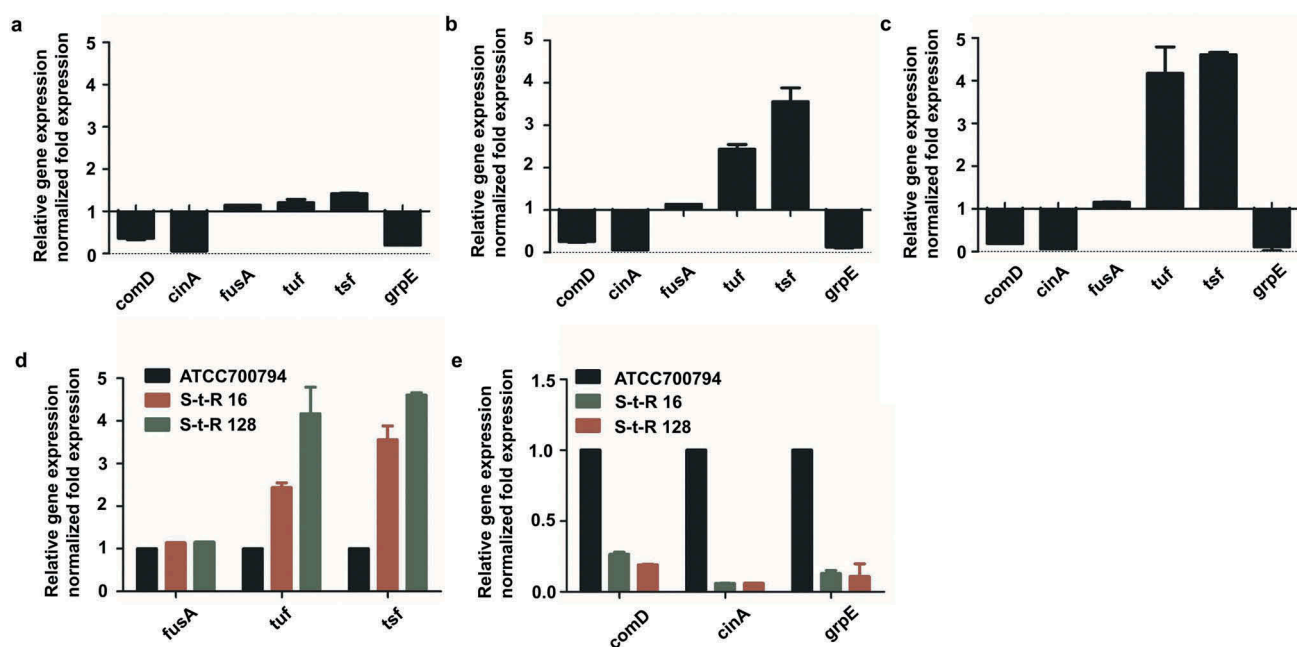
**Figure 3.** The network of significantly differentially expressed proteins (ratio >1.2 or <0.8 ( $p$ -value < 0.05)) was analyzed by STRING. Small nodes represent proteins of unknown 3D structure; large nodes represent proteins with some 3D structure information or predicted. Coloured nodes represent query proteins and first shell of interactors; white nodes represent second shell of interactors. The blue lines represent database evidence; the purple lines represent experimental evidence; yellow lines represent text mining evidence; the black lines represent coexpression evidence; and green lines represent neighbourhood evidence.

**Table 3.** MICs of resistance strain *Streptococcus suis* ATCC 700794.

Antibiotic	Sensitive strain (ATCC 700794)	Resistance strain (S-t-R 128)		CLSI		
tetracycline	0.5 µg/mL	128 µg/mL	S	2	R	8
ofloxacin	1 µg/mL	32 µg/mL	S	2	R	8
enrofloxacin	0.5 µg/mL	2 µg/mL	S	0.5	R	2
chlortetracycline	0.625 µg/mL	32 µg/mL	S	2	R	8
florfenicol	2 µg/mL	2 µg/mL	S	2	R	8
penicillin K	1 µg/mL	1 µg/mL	S	0.5	R	2

understanding of proteins that are differentially expressed under tylosin stress.

In order to explore the differences in protein expression treated with 0.125 µg/mL tylosin and non-untreated *S. suis*,



**Figure 4.** (a). Effect of 1/4 MIC of tylosin on mRNA expression of genes in *Streptococcus suis* ATCC 700794. Data are expressed as means  $\pm$  standard deviations. The expression was normalized to 16S rRNA. Significantly different ( $*p < 0.05$ ) compared to untreated control bacteria. (b). Effect on mRNA expression of genes in drug resistance level at 16  $\mu\text{g}/\text{mL}$  of tylosin (S-t-R 16) *Streptococcus suis* ATCC 700794. Data are expressed as means  $\pm$  standard deviations. The expression was normalized to 16S rRNA. Significantly different ( $*p < 0.05$ ) compared to control bacteria. (c). Effect on mRNA expression of genes in drug resistance level at 128  $\mu\text{g}/\text{mL}$  of tylosin (S-t-R 128) *Streptococcus suis* ATCC 700794. Significantly different ( $*p < 0.05$ ) compared to untreated control bacteria. Data are expressed as means  $\pm$  standard deviations. The expression was normalized to 16S rRNA. Significantly different ( $*p < 0.05$ ) compared to control bacteria. (d). Effect on mRNA expression of *fusA*, *tuf*, *tsf* in *Streptococcus suis* ATCC 700794, drug resistance level at 16  $\mu\text{g}/\text{mL}$  of tylosin (S-t-R 16) *Streptococcus suis* ATCC 700794 and drug resistance level at 128  $\mu\text{g}/\text{mL}$  of tylosin (S-t-R 128) *Streptococcus suis* ATCC 700794. Data are expressed as means  $\pm$  standard deviations. The expression was normalized to 16S rRNA. Significantly different ( $*p < 0.05$ ) compared to control bacteria. (e). Effect on mRNA expression of *comD*, *cinA*, *grpE* in *Streptococcus suis* ATCC 700794, drug resistance level at 16  $\mu\text{g}/\text{mL}$  of tylosin (S-t-R 16) *Streptococcus suis* ATCC 700794 and drug resistance level at 128  $\mu\text{g}/\text{mL}$  of tylosin (S-t-R 128) *Streptococcus suis* ATCC 700794. Data are expressed as means  $\pm$  standard deviations. The expression was normalized to 16S rRNA. Significantly different ( $*p < 0.05$ ) compared to control bacteria.

we observed proteins mainly belonging to protein elongation factor, two-component signal transduction system (TCS) and heat shock protein families based on our functional analysis using STRING. Previous studies suggest three types of elongation factors that required for prokaryotic cells translation are involved in drug resistance. Among them, the translation elongation Factor-G (EF-G) was reported to increase the resistance to kanamycin in *E. coli* via different point mutations in EF-G [24,45]. EF-Tu, another elongation factor, is the target of four families of antibiotics viz kirromycin, enacyloxin IIa, pulvomycin and GE2270A in *E. coli* [46,47]. Additionally, EF-G and EF-Tu have been identified as up-regulated proteins in tylosin-resistant *M. gallisepticum* [27]. EF-TS has been displayed a significant change in the multidrug resistant *E. coli* strains [23]. To find out whether these proteins were related to drug resistance in *S. suis*, strains S-t-R 16 and S-t-R 128 were induced in our experiment. The genes *fusA* (EF-G), *tuf* (EF-Tu) and *tsf* (EF-Ts) were up-regulated in strains S-t-R 16 and S-t-R 128, compared to the *S. suis* ATCC

700794. The expression of these elongation factor genes changed significantly with increase in the degree of resistance. This could be attributed to the function of tylosin. Previous research has reported that the MIC of ofloxacin is up-regulated in erythromycin-induced strain [48] and the MIC of chlortetracycline increased 64 times in the tylosin-induced *E. coli* [49]. In our experiment, the MICs of terramycin, chlortetracycline, ofloxacin and enrofloxacin were shown high-level resistance in the S-t-R 128 strain. Some studies have provided evidence that the “site” mutations of EF-G result in large changes in gene expression such as *cyaA* (adenylate cyclase) and *topA* (topoisomerase I) [24]. The *topA* gene acts jointly with topoisomerase IV genes (*parC* and *parE*) and relaxes DNA supercoils in opposition to GyrAB activity, while the GyrAB protein is the most important site to form the resistance of fluoroquinolone [21]. Previous studies have reported that the tetracycline (TC) antibiotics inhibit bacterial protein synthesis by preventing the attachment of aminoacyl tRNA to the ribosomal acceptor A site [50]. The S-t-R 128 strain showed

multidrug resistance to macrolides, quinolones and tetracycline that means the protein elongation factors may play important roles in conferring resistance to quinolones and tetracycline.

Natural transformation is a broadly conserved mechanism for horizontal gene transfer in bacterial species [51]. In *S. pneumoniae*, several observations suggest that mosaic *PBP* genes was taken up at the physiological state. This state is referred to as competence. Bacteria belonging to the *Streptococcus* genus, such as *S. pneumoniae*, *Streptococcus mutans* and *S. suis* are reported to be transformable species [46,52]. The TCS pathway, ComCDE, is involved in controlling competence. ComD and putative competence-damage inducible protein, which were significantly down-regulated in tylosin-treated sample, belong to the TCS of ComCDE.

In our study, *comD* and *cinA* gene transcripts were significantly down-regulated which was in agreement with to the proteomics data. Similar results were obtained in tylosin-resistant strain in which the expression levels of *cinA* and *comD* were significantly down-regulated. Additionally, down-regulation of these genes correlated with the level of drug resistance due to tylosin. Since, *S. suis* has been reported as the transformable species, ComCDE may exist in *S. suis* and play an important role in multidrug resistance.

A heat shock protein in *Stenotrophomonas maltophilia* acted as a stress-responsive protein by inducing quinolone resistance [25]. Additionally, a study demonstrates role of the heat shock protein GrpE that has proved early exposure to these drugs in *E. coli* [53]. In this study, GrpE was significantly down-regulated in tylosin resistant *S. suis* and this correlated with the level of drug resistance. Previous studies have also shown that expression levels of GrpE in tylosin resistant *M. gallisepticum* were significantly altered at various drug concentrations [27]. Taken together, it is possible that GrpE may be involved in multidrug resistance.

We analyzed differentially expressed proteins of *S. suis* in response to tylosin stress by iTRAQ-based proteomic analysis. Several proteins showed significant changes in their level of expression upon tylosin stress in *S. suis*, including protein elongation factor, TCS and GrpE. Six drug resistance related proteins were determined in tylosin-resistant *S. suis* in response to induction with the drug and the corresponding changes in transcript levels were determined. The results showed that protein translation, elongation factor G, elongation factor Tu, elongation factor Ts, putative histidine kinase of the competence regulon, ComD, putative competence-damage inducible protein and protein GrpE were differentially expressed. The MICs of tetracycline and fluoroquinolone showed higher level of resistance compared to

the sensitive bacteria. Based on these observations, we infer that these variations may be due to stress from the tylosin. Our data would help in identifying new target responsible for tylosin-resistant in *S. suis* and reducing antibiotic resistance by corresponding inhibitors.

## Abbreviations

*S. suis* *Streptococcus suis*  
MIC Minimum inhibitory concentration  
iTRAQ isobaric tag for relative and absolute quantitation

## Author contributions

Y-HL designed the whole experiment. R-XC directed the completion of the experiment. X-XX, XL, Q-WQ, MC, FY, J-XM, X-RC, Y-HZ, B-OG, J-WL, Z-XL, Y-PX were supportive during the experiment.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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