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Comparative proteome analysis of two *Streptococcus agalactiae* strains from cultured tilapia with different virulence

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ABSTRACT

Streptococcus agalactiae is a major piscine pathogen, which causes significant morbidity and mortality among numerous fish species, and results in huge economic losses to aquaculture. Many *S. agalactiae* strains showing different virulence characteristics have been isolated from infected tilapia in different geographical regions throughout South China in the recent years, including natural attenuated *S. agalactiae* strain TFJ0901 and virulent *S. agalactiae* strain THN0901. In the present study, survival of tilapia challenged with *S. agalactiae* strain TFJ0901 and THN0901 (10^7 CFU/fish) were 93.3% and 13.3%, respectively. Moreover, there are severe lesions of the examined tissues in tilapia infected with strain THN0901, but no significant histopathological changes were observed in tilapia infected with the strain TFJ0901. In order to elucidate the factors responsible for the invasive potential of *S. agalactiae* between two strains TFJ0901 and THN0901, a comparative proteome analysis was applied to identify the different protein expression profiles between the two strains. 506 and 508 cellular protein spots of *S. agalactiae* TFJ0901 and THN0901 were separated by two dimensional electrophoresis, respectively. And 34 strain-specific spots, corresponding to 27 proteins, were identified successfully by MALDI-TOF mass spectrometry. Among them, 23 proteins presented exclusively in *S. agalactiae* TFJ0901 or THN0901, and the other 4 proteins presented in different isomeric forms between TFJ0901 and THN0901. Most of the strain-specific proteins were just involved in metabolic pathways, while 7 of them were presumed to be responsible for the virulence differences of *S. agalactiae* strain TFJ0901 and THN0901, including molecular chaperone DnaJ, dihydrolipoamide dehydrogenase, thioredoxin, manganese-dependent inorganic pyrophosphatase, elongation factor Tu, bleomycin resistance protein and cell division protein DivIVA. These virulence-associated proteins may contribute to identify new diagnostic markers and help to understand the pathogenesis of *S. agalactiae*.

1. Introduction

Streptococcus agalactiae is a major piscine pathogen responsible for huge economic losses to aquaculture. It causes significant morbidity and mortality among numerous fish species of freshwater, estuarine and marine,

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including gulf killifish, tilapia, silver pomfret, barcoo grunter and grouper (Liu et al., 2013; Pridgeon and Klesius, 2013). Clinical signs of *S. agalactiae* disease include septicemic infection, exophthalmia, corneal opacity, anorexia, 'C'-shaped body posturing and erratic swimming (Ye et al., 2011).

Tilapia production in the southern provinces of China, such as Guangdong, Guangxi, Fujian and Hainan, accounted for almost 40% of the world. However, the large scale disease outbreaks caused by *S. agalactiae* has brought a great loss in economic terms. The first isolation of *S. agalactiae* from tilapia in China was reported in Fujian Province, where it caused 20–30% mortality of tilapia in limited areas. Afterward, disease outbreaks spread rapidly to other tilapia farms in major cultivation areas of Southern China, especially in Guangdong and Hainan Provinces, and the mortality rates of over 95% were observed in some farms (Ye et al., 2011). In our previous studies, many *S. agalactiae* strains were isolated from infected fish in different geographical regions throughout South China (Li et al., 2013; Liu et al., 2013), which presented different virulence characteristics. Among them, a natural attenuated strain TFJ0901, isolated from infected tilapia in Fujian, caused no significant symptom and extremely low mortality in tilapia. There must be some unknown factors associated with the attenuated virulence characteristics for us to further study.

Some virulence factors of *S. agalactiae* were well characterized in human, such as polysaccharide capsule, β-hemolysin/cytolysin, Srr1, nuclease A and other surface proteins that mediate binding to host cells, extracellular matrix, and blood components (Derré-Bobillot et al., 2013; Lindahl et al., 2005; Seo et al., 2012). However, bacterial pathogens have developed such a variety of strategies to infect hosts requiring the involvement of large quantities of virulence associated factors, including bacterial adherence and invasion to the host cells, and replication inside the host to lead to the final cell death (Fittipaldi et al., 2012). The previous studies on the virulence associated factors of *S. agalactiae* were far from enough, so it does make sense for us to explore more proteins involved in pathogenesis. Combining two-dimensional electrophoresis with mass spectrometry resulted in a powerful technology ideally suited to recognize and identify proteins of pathogenic microorganisms (Cordwell et al., 2001). The proteomics study using *S. agalactiae* cells grown under different conditions has been reported, and C protein ß antigen was proposed to be a putative virulence factor (Yang et al., 2010). Hence, comparative proteome analysis was applied in our study to illustrate different protein expression profiles between the natural attenuated *S. agalactiae* strain TFJ0901 and virulent *S. agalactiae* strain THN0901 and the differentially expressed proteins may contribute to the pathogenesis of *S. agalactiae*.

2. Materials and methods

2.1. S. agalactiae strains with different virulence

Five *S. agalactiae* strains used in this research were originally isolated from infected tilapia during the outbreaks of *S. agalactiae* throughout South China, and they were named TFJ0901, TMM1101, TZH1201, TZC1101, and THN0901, respectively. Infection chronology, sources of isolation, hosts and serotypes of the *S. agalactiae* strains were summarized in Table S1. *S. agalactiae* strains were grown aerobically overnight at 28 °C in a shaker bath, and then overnight cultured cells were diluted into 1:100 in BHI medium. Tilapia (*Oreochromis niloticus*) for this experiment were purchased from a breeding farm in Guangdong province, with mean weights of 76 ± 5 g. They were acclimated for two weeks before grouping (28–30 °C). A total of 30 fish were used in each treatment group (15 fish per tank, duplicates), and two replicate tanks of tilapia served as controls. The same amount (10^7 CFU/fish in a total volume of 0.1 mL) (Pridgeon and Klesius, 2013) of each *S. agalactiae* strain was exposed to the tilapia via intraperitoneal injection. Survivals of the 6 groups were recorded daily for 14 days. Meanwhile, brains, kidneys and spleens of tilapia infected by *S. agalactiae* strains were sampled after 7 days post inoculation (dpi). Following standard fixation in 10% neutral buffered formalin and processing into paraffin wax blocks, paraffin sections (5 μm thick) were stained with hematoxylin and eosin (H&E) for light microscopy observations. Approval was obtained from the animal ethics committee of the life science institute prior to using the animals for research.

2.2. Preparation of cellular proteins

S. agalactiae strains THN0901 and TFJ0901 were grown aerobically overnight at 28 °C in a shaker bath, and then overnight cultured cells were diluted into 1:100 in BHI medium. Optical densities at 600 nm were measured and the growth curve of bacterial cultures was presented as Fig. S1. The cultures were collected at early stationary (12 h) by centrifugation at 10,000 × *g* for 10 min at 4 °C and were washed by ddH_2O for 3 times. Then the pellets were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS), and sonicated on ice with the ultrasound power of 75 W for 30 min, worked for 4 s and stopped for 6 s in a cycle. The supernatant was collected by centrifugation at 12,000 × *g* for 20 min at 4 °C and 100% trichloroacetic acid was added to a final concentration of 10%, kept at 4 °C for 4 h. The pellets were collected, and ice–cold acetone was added to wash it. After the pellets were centrifuged at 12,000 × *g* for 20 min at 4 °C and dissolved in lysis buffer, the concentration of the soluble protein in the final preparation was determined by the Bradford method and BSA was used as standard protein.

2.3. Two-dimensional electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed as previously described (Boguth et al., 2000). 800 μg cellular proteins were diluted to a total volume of 350 μL with rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS and 0.005% (w/v) bromophenolblue, 1% (v/v) IPG buffer 3–10), and the proteins were separated on 17 cm pH 4–7 NL ReadyStrips™ IPG Strips (Bio-rad). IEF was performed with a PROTEAN® IEF cell using the program: 50 V active rehydration for 12 h; 150 V (rapid ramp), 150 Vh; 300 V (rapid ramp), 300 Vh; 500 V (rapid ramp),