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Development of an attenuated oral vaccine strain of tilapia Group B Streptococci serotype Ia by gene knockout technology

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ABSTRACT

Our previous studies demonstrated that the deletion of D2 fragment in tilapia *Streptococcus agalactiae*(GBS) attenuated strain YM001 is the main reason for the loss of virulence to tilapia. In this study, a Δ2 mutant that deletion of D2 fragment in parental virulent strain HN016 was constructed, and the safety, stability, immunogenicity, and growth characteristics, as well as the virulence mechanism of Δ2 mutant were evaluated. The results showed that Δ2 mutant was not pathogenic to tilapia, and the virulent revertants were not observed after 50 generations of passage. The RPS reached 96.11% at 15 days and 93.05% at 30 days, respectively, after intraperitoneal injection, while RPS reached 74.80% at 15 days and 53.16% at 30 days, respectively, after oral immunization. The growth of Δ2 mutant was significantly faster than YM001, and genes that were enriched in the nitrogen metabolism and arginine biosynthesis signaling pathway (arc, glnA, and gdhA) were identified as important candidate genes responsible for growth rate of *S. agalactiae.* The absence of D2 fragment affected the expression of Sip, therefore influencing the bacterial virulence. Altogether, this study demonstrated that deletion of D2 fragment in HN016 causes the loss of virulence to tilapia, and Δ2 mutant is a promising, better attenuated oral vaccine strain of *S. agalactiae* compared to YM001.

1. Introduction

Streptococcus agalactiae is one of the most important pathogens causing fish streptococcosis, which can induce death in a variety of seawater and freshwater farmed fish [1–5]. Since 2009, the Chinese tilapia aquaculture industry has experienced a large-scale streptococcal epidemic, with a mortality rate of 30%–90%, and more than 90% of the clinical strains are *S.agalactiae* [6–10]. At present, antibiotics are mainly used for the treatment and prevention of fish streptococci, but the problems caused by environmental pollution, drug residues and drug-resistant strains are serious and unacceptable. Therefore, vaccines are generally recognized as the most ideal alternative [11,12]. Among various available vaccines, attenuated oral vaccine is the most ideal one, which has advantages of convenient vaccination, less stressful to fish, and broad inoculation range [13,14], and stimulates a more robust humoral and cell-mediated immune response than inactivated bacteria or subunit vaccines in fish [15].

The key thing to prepare an oral attenuated vaccine is to obtain a suitable attenuated vaccine strain. At present, attenuated strains are mainly prepared by two methods. One is to use low-dose antibiotics to screen resistant strains, but this approach carries the risk that bacteria will revert their original virulence. For example, Pridgeon et al. obtained an attenuated *S. agalactiae* strain by screening at different concentrations of sparfloxain, but the strain still has some virulence to tilapia, and its virulence was reverted after removing antibiotics. In addition, injection was required for this vaccine [16]. Another method is to knock out the specific genes of pathogens by techniques such as random transposon mutagenesis or gene recombination, but the drawback of the method is that there is a lack of stability for the obtained strain. For example, Huang et al. constructed a *S. agalactiae* recombinant DNA vaccine by using attenuated *Salmonella typhimurium* SL7207 as a vector, which can be orally immunized. However, the attenuated strain produced by this method has poor stability and weak immune response, and immunoprotection can only last 7 days [17]. Continuous *in vitro* passage is a classic method for obtaining attenuated bacterial strains. Compared with the above two methods, although a large amount of preparation time and labor cost are required, attenuated strains from continuous *in vitro* passage exhibit significantly improved stability and immunogenicity [18]. So far, the attenuated *S. suis* strain ST171 obtained by continuous *in vitro* passage in 1978 was still widely used in China [19]. We have previously prepared tilapia-derived, an attenuated *S. agalactiae* vaccine strain YM001 (Ia, ST7) by

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this method, which shows good safety, stability and immune effect, and can be orally immunized [20]. However, YM001 shows lower growth rate than its parental strain HN016 (Ia, ST7), which limits its application in practice.

Our previous studies showed that YM001 has two large fragment deletions (D1, 5, 832 bp; D2, 11, 116 bp) compared to HN016, resulting in the deletion of three rRNAs and ten tRNA genes, as well as deletion and function defects of 10 genes involved in metabolism, transport, growth, anti-stress, etc [21]. It has been speculated that the deletion of the D2 fragment is the main reason causing the loss of virulence to tilapia for YM001. In this study, a Δ2 mutant was obtained by knockout of D2 fragment in HN016, and the effects of D2 fragment on the virulence of *S. agalactiae* strain were verified by multi-omics analysis, animal experiments and RT-qPCR. Moreover, the growth characteristic, safety, stability and immunogenicity of the knockout strain were analyzed in order to evaluated its usefulness as a vaccine candidate. Our study provides theoretical basis for the development of other *S. agalactiae* vaccines in future.

2. Materials and methods

2.1. Bacterial strains and fishes

The *S.agalactiae* strain HN016 was isolated from a moribund cultured tilapia with typical clinical and pathogenic characteristics of meningitis (Hainan, China, 2010). The HN016 strain was used as starting material to generate an attenuated strain by continuous passage *in vitro*, and the 840th passage was named strain YM001. The D2 deletion sequence from YM001 was confirmed by previous genome study [20] and a D2 deletion mutant in HN016 was further constructed by homologous sequence replacement technology. The serotype and ST type of YM001 and △2 mutant strains were consistent with HN016(Ia,ST7) [20].

Non-infected Nile tilapia(*Oreochromis niloticus*) with average weight of 400 ± 33.26 g were provided by the National Tilapia Seed Farm (Nanning, Guangxi, China), which were verified to be negative for bacterial infection by bacteriological analysis of the brain and kidney samples. The fishes were monitored twiced a day with a formulated diet (Tongwei Feed Company, Nanning, China).

2.2. Growth assays

The strains were cultured as we described previously [20]. Briefly, the strains stored at −80 °C were streaked onto 5.0% sheep blood agar plates, and incubated at 28 °C for 24 h, and the morphology of each strain was observed under a microscope and a scanning electron microscope (TESCAN, VEGA3LMU, Czech). The recovered strains were then inoculated into 500 mL modified Martin medium at 28 °C by shaking, and the viable colony-forming units (CFUs) of the liquid culture was determined at 0 h, 4 h, 8 h, 12 h, 16 h, 24 h, 48 h, 72 h, respectively. The bacterial density (CFU/mL) was determined by plating 100 μl of 10-fold serially-diluted culture onto sheep blood agar plates and counting of the colonies. Then collected the bacterium solution and stored in RNAprotect®bacteria Reagent(QIAGEN, Germany), then sent to the Novogene Co, LTD (Beijing) for RNA-seq and proteome analysis.

2.3. Construction of gene knockout mutant

A primer pair of the target gene was designed and the gene was amplified using high-fidelity PCR enzyme, and further purified by Gel purification. The suicide plasmid vector pSET4s was constructed using plasmid pSET4s supplied by Biovector NTCC Inc. The pSET4s was isolated and digested with restriction enzymes HindIII and EcoRI at 37 °C for 2 h,and then further purified. The ligation was performed by using ClonExpress Entry One Step Cloning Kit (Vazyme Biotech Co.,Ltd) according to the manufacturer's instruction. The ligation mixture was transformed into the *E.coli*, 6 transformants were randomly picked up and the correct clones were validated by PCR amplification. The HN016 competent cells were prepared by using the method described by Takamatsu et al. [22]. The competent bacteria were transformed by electroporation. After confirming the single exchange, the culture was continuously passed at 28 °C to screen the clones with double exchange, and further verified by colony PCR. The primers used in the experiments were listed in Table S1.

2.4. Virulence test of △2 mutant

Comparising the virulence to tilapia between YM001, △2 mutant and HN016 strains by IP injection(eight dosages) and oral gavage(two doses). The strains were cultured by our previous methods [20]. After anesthesia by immersion into a bath of 10 mg/L benzocaine (Sigma, USA), a total of 40 fishes were given HN016, △2 mutant or YM001 at each dose by IP injection (0.1 mL/fish and 20 fishes/tank, with 2 replicates). The control group was injected with 0.1 mL of sterile TSB. Meanwhile, in oral gavage group, each fish was administered with 0.5 mL of bacterial culture by oral gavage, and the control group was treated with 0.5 mL of TSB. The infected fishes were monitored and fed twice a day for 21 days, and the bacteria were re-isolated from the brain and liver tissues of all deadfishes at the end of the experiment and identified. The experiment was conducted twice.

The brain, liver, spleen, head kidney, and intestine of infected tilapia were collected for tissue injury pathological analysis, Samples were collected from the freshly dead fish. For live fish group, the animals were sacrificed with high concentration of benzocaine before the tissues were collected. Following standard fixation in 10% neutral buffered formalin and sample processing in paraffin wax blocks, paraffin sections (6 μm thick) were stained with hematoxylin and eosin (H &E) for light microscopy observations.

2.5. Back-passage safety studies with △2 mutant

A back-passage safety of △2 mutant was performed by our previous published methods [20]. Briefly, 150 fishes in total were equally devided into control and vaccine groups with 50 per group, which were then devided into 10 tanks with 15 fish per tank. The fishes in tank NO.1 received △2 mutant vaccine (1.0×10^9 CFU/200μL/fish) via IP injection, while the control group was injected with 0.2 mL of TSB. After 48 h, 5 fishes were taken from the first tank and sacrificed, and cultured the bacteria on blood agar plate which isolated from the brains, then the bacteria (0.2 mL, 1.0×10^9 CFU/200μL/fish) was then injected into the fishes in tank No. 2. This procedure was repeated 10 times with the remaining fishes, and the mortality or adverse behavior or signs of disease were recorded daily for 21 days post injection.

Then repeat the back-passage procedure above for 5 times until get the 50th passage of the △2 mutant.

2.6. Immunogenicity assay

A total of 200 fishes were equally devided into two groups(100/group and 50/tank, with 2 replicates), one was given △2 mutant by IP injection and another served as bland control. The injection group received △2 mutant (1.0×10^8 CFU/0.1 mL/fish) via IP injection, while the control group injected with 0.1 mL PBS per fish. For HN016 challenge, the 100 fishes in each group were divided into two subgroups (50/subgroup and 25/tank, with two replicates). The two subgroups were given with HN016 (1.0×10^6 CFU/fish, 100 LD50) by IP injection after 15 and 30 days of △2 mutant vaccination, respectively, and the challenged fishes were monitored and fed twice a day for 15 days. Relative percent survival (RPS) = {1 - (vaccinated mortality ÷ control mortality)}100. The test was repeated twice.

2.7. RNA-sequencing

The stored strains were sent to the Novogene Co, LTD (Beijing, China) for RNA-seq. All following Kits listed were used according manufacture's recommendations. Total RNA was extracted by using Tiangen RNA prep Pure Plant Kit (Tiangen Biomart, Beijing). RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA,USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations [23] and index codes were added to attribute sequences to each sample. Quantified cDNA libraries (effective concentration, 2 nM) were sequenced using an Illumina HiSeq platform. Clean reads were obtained by removing low-quality reads, and reads containing poly-N and adapters were mapped back onto the reference genome sequence (NCBI, Accession NO.NZ_CP011325.1) by using STAR (v2.5.1b). HTSeq v0.6.0 was used to count the reads numbers mapped to each gene [24]. Transcript abundance was measured as a unit of the expected number of fragments per kilobase of transcript per million mapped reads (FPKM) [25]. Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (1.10.1) [26]. DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value < 0.05 found by DESeq2 were assigned as differentially expressed. The statistical enrichment of DEGs in Gene Ontology was performed using GOseq [27], DEGs in KEGG pathways was performed using KOBAS v2.0 [28]. Biotechnology Information(NCBI) with the accession numbers < SRR7841403 > , < SRR7841410 > and < SRR7841481 > for YM001, HN016 and △2 mutant, respectively.

2.8. Protein extraction and peptide preparation

The samples were individually milled to a power in a mortar with liquid nitrogen. We then mixed 150 mg of the powder from each sample with 1 ml of lysis buffer containing Tris-base (pH 8), 8 M Urea, 1% SDS, complete protease inhibitor cocktail (Sigma) in a glass homogenizer. The homogenate was incubated on ice for 20 min and then centrifuged at 12000 g for 15 min at 4 °C. The supernatant was transferred to a clean tube, and protein concentration was determined with a Bradford assay. And then added 4 vol 10 mM DTT in cold acetone to a sample extract, vortexed well, placed samples at −20°Cfor 2 h or overnight. Centrifuged and collected pellet to wash twice with cold acetone. Finally dissolved the pellet by dissolution buffer containing Tris-base (pH = 8), 8 M Urea.

2.9. iTRAQ labeling, HPLC fractionation and LC-MS/MS analysis

Desalted peptides were labeled with iTRAQ reagents (iTRAQ® Reagent-8PLEX Multiplex Kit,Sigma), following the manufacturer's instructions (AB Sciex, Foster City, CA). For 0.1 mg of peptides, 1 unit of labeling reagent was used. Peptides were dissolved in 20 μl of 0.5 M triethylammonium bicarbonate solution (TEAB, pH 8.5), and the labeling reagent was added to 70 μl of isopropanol. After incubation for 1 h, the reaction was stopped with 50 mM Tris/HCl (pH 7.5). Differently labeled peptides were mixed equally and then desalted in 100 mg SCX columns (strata-x-c, Phenomenex: 8B–S029-EBJ).

A 600 μg iTRAQ-labeled peptide mix was fractionated using a C18 column (waters BEHC18 4.6 × 250 mm, 5 μm) on a Rigol L3000 HPLC operating at 1 ml/min. The column oven was set as 50 °C. Mobile phases A (2% acetonitrile, 20 mM NH4FA, adjusted pH to 10.0 using NH3·H2O) and B (98% acetonitrile, 20 mM NH4FA, adjusted pH to 10.0 using NH3·H2O) were used to develop a gradient elution. The solvent gradient was set as follows: 3–8% B, 5min。 8–18% B, 12 min。 18–32% B, 11 min。 32–45% B, 7 min。 45–80% B, 3 min。 80% B, 5 min。 80–5%, 0.1min, 5% B, 7 min. The tryptic peptides were monitored at UV 214 nm. Eluent was collected every minute and then merged to 15 fractions. The samples were dried under vacuum and reconstituted in 20 μl of 0.1% (v/v) FA, 3% (v/v) acetonitrile in water for subsequent analyses.

Fractions from the first dimension RPLC were dissolved with loading buffer and then separated by a C18 column (150 μm inner-diameter, 360 μm outer-diameter × 15 cm, 1.9 μm C18, Reprosil-AQ Pur, Dr. Maisch). Mobile phase A consisted of 0.1% formic acid in water solution, and mobile phase B consisted of 0.1% formic acid in acetonitrile solution。 a series of adjusted 60 min gradients according to the hydrophobicity of fractions eluted in 1D LC with a flow rate of 300 nL/min was applied. Q-Exactive HF-X mass spectrometer was operated in positive polarity mode with capillary temperature of 320 °C. Full MS scan resolution was set to 60000 with AGC target value of 3e6 for a scan range of 350–1500 *m/z*. A data-dependent top 40 method was operated during witch HCD spectra was obtained at 15000 MS2 resolution with AGC target of 1e5 and maximum IT of 45 ms, 1.6 *m/z* isolation window, and NCE of 30, dynamically excluded of 60s.

2.10. The identification and quantitation of protein

The resulting spectra from each fraction were searched separately against the "Run2_Streptococcus_agalactiae_GCF_001190805.1_ASM119080v1_protein.fasta" database by Proteome Discoverer2.2 software(Thermo Fisher Scientific). The searched parameters as follows: A mass tolerance of 10 ppm for precursor ion scans and a mass tolerance of 0.02 Da for the product ion scans were used. Carbamidomethyl was specified in PD 2.2 as fixed modifications. Oxidation of methionine, acetylation of the N-terminus and iTRAQ 8-plex of tyrosine, lysine were specified in PD 2.2 as variable modifications. A maximum of 2 miscleavage sites were allowed.

For protein identification, protein with at least 1 unique peptide was identified at FDR less than 1.0% on peptide and protein level, respectively. Proteins containing similar peptides and could not be distinguished based on MS/MS analysis were grouped separately as protein groups. Reporter Quantification (iTRAQ 8-plex) was used for iTRAQ quantification. The protein quantitation results were statistically analyzed by Mann-Whitney Test, the significant ratios, defined as $p < 0.05$ and $|\log2FC| > *$(ratio > * or ratio < * [fold change, FC]), were used to screen the differentially expressed proteins (DEP).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the MASSIVE with the dataset identifier < PXD011206 > .The reviewer account details see additional file:Table S2.

2.11. Data analysis of proteome

Gene Ontology (GO) and InterPro (IPR) analysis were conducted using the interproscan-5 program against the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART, ProSiteProfiles, PANTHER) [29], and the databases COG (Clusters of Orthologous Groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes) were used to analyze the protein family and pathway. The probable interacting partners were predicted using the STRING-db server (http://string.embl.de/) based on the related species. STRING is a database of both known and predicted protein-protein interactions [30]. The enrichment pipeline [31] was used to perform the enrichment analysis of GO, IPR, COG and KEGG, respectively.

2.12. Validation of gene expression level by real-time quantitative PCR (RT-qPCR)

The RT-qPCR was used to verify the expression levels of the candidate genes between YM001, △2 mutant and HN016 in culture strains and different infected tilapia tissues (Fig. 3C). The recA [32] gene was selected as a standardization control, and the specific primers used to amplify the candidate genes were designed using Primer 5 software. Briefly, total RNA was extracted respective from HN016,△2 mutant and YM001 strains, and infected tilapia tissues (brain, liver, spleen, head kidney, and intestine, respectively). then reverse transcribed into cDNA by HiScript® II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing). Real-time qPCR was performed in a DNA Engine Chromo 4 real-time system (BioRad) with HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme, Nanjing). The expression of genes was calculated as relative expression to recA using the 2(-Δ Δ C(T)) method and samples were analyzed in triplicates.

3. Results

3.1. Construction of D2 deletion mutant

To evaluate the role of the D2 fragment in HN016, Δ2 mutant was constructed by homology recombination (Fig. 1). The D2 fragment knockout strain was identified by PCR. The results indicated that the upstream and downstream homology arms of the D2 fragment (Fig. 1A), the gene knockout plasmids (Fig. 1B and C) and transformants (Fig. 1D) were correctly constructed. The expression of the D2 fragment was not detected in the Δ2 mutant (Fig. 1D), demonstrating that the D2 fragment knockout strain of HN016 has been successfully constructed.

3.2. Growth assays

The growth rate of Δ2 mutant was slightly lower than that of HN016

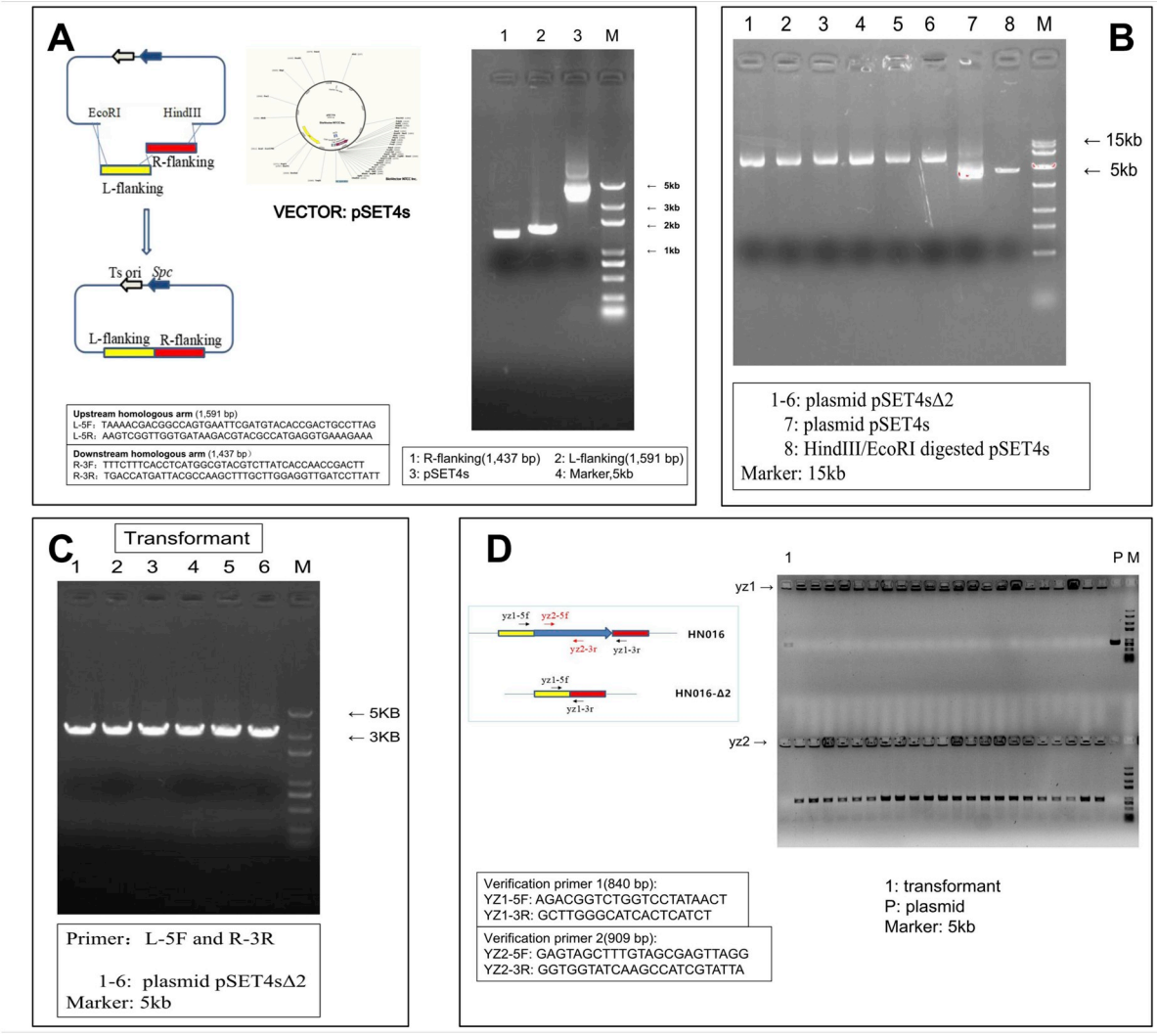


Fig. 1. Homologous sequence replacement technique was used to construct the D2 fragment knockout strain of HN016. A showes the construction of the upstream and downstream homologous arms. B and C showed the construction of plasmid. D showed the construction of the transformant.

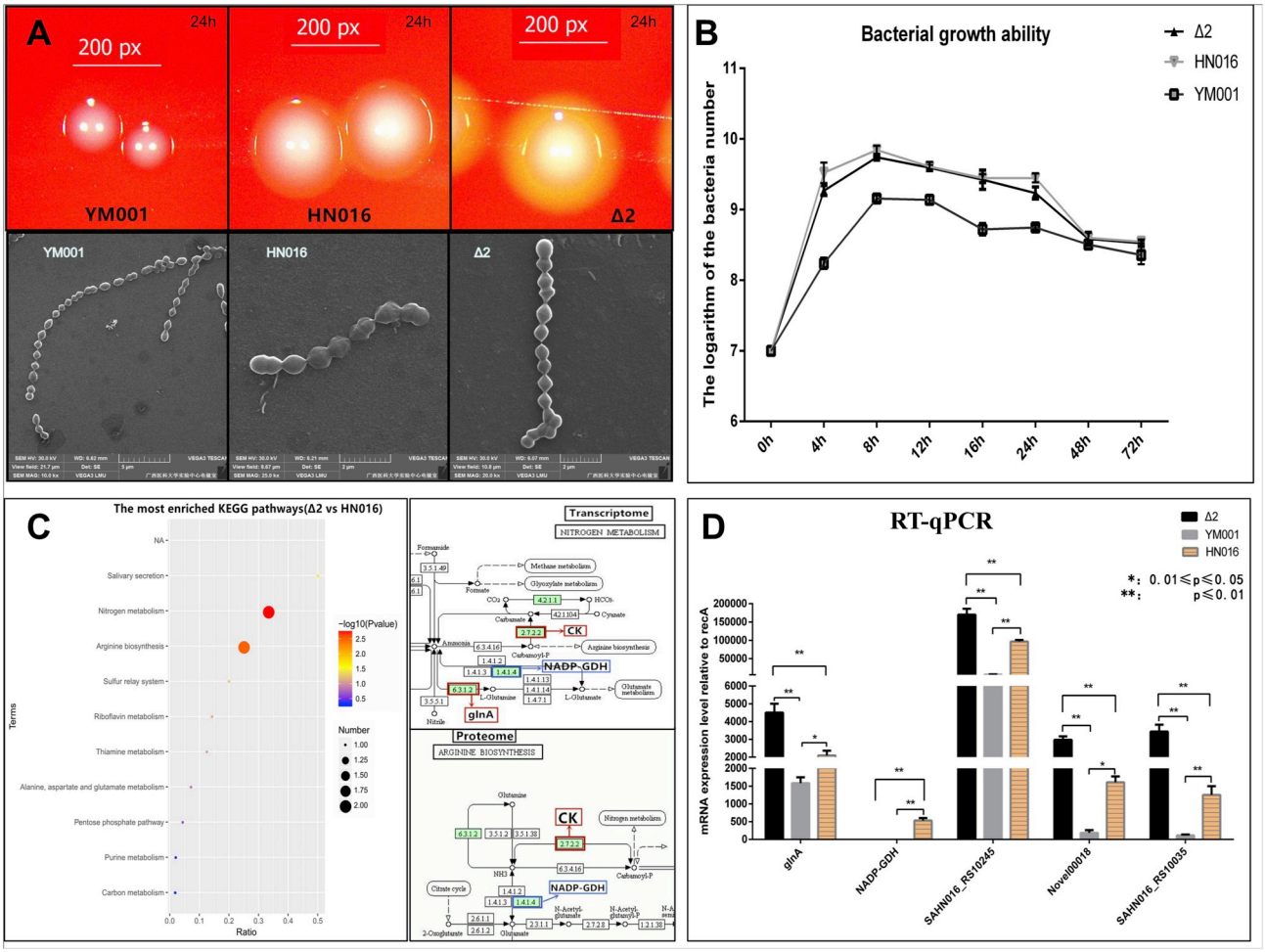


Fig. 2. The growth ability of the strains. A shows the morphological characters of the HN016, YM001 and Δ2 mutant when observed under microscope and scanning electron microscope. B shows the growth curve of the strains. C shows the omics analysis of the strains. D shows the RT-qPCR verification result.

but significantly higher than that of YM001 (Fig. 2B) when all three strains were grown in modified Martin medium at 28 °C. The morphology of each strain was observed under a microscope, and the bacterial size of HN016 and Δ2 mutant was significantly larger than that of YM001. HN016 and Δ2 mutant showed obvious hemolysis, but YM001 has no obvious hemolysis. After 4 h, there was no significant change in the size of all three strains. The morphology of each strain was further observed under scanning electron microscope. The chain length of YM001 was significantly longer than that of HN016 and Δ2 mutant, followed by Δ2 mutant, and HN016 was the shortest; YM001 was rarely single chains, while Δ2 mutant and HN016 appeared mostly short and single chains (Fig. 2A).

3.3. The results of virulence, back-passage safety and immunogenicity tests

Tilipia were administered with YM001, HN016, and Δ2 mutant by IP injection and oral gavage, respectively. The virulence test results (Table 1) showed that the LD50 (lethal dose, 50%) of injected HN016 was approximately 5.4×10^4 CFU/fish, while the mortality rates in fishes received high-dose of HN016 (1.0×10^9 and 1.0×10^{10} CFU/

Table 1
Virulence of YM001, △2 mutant and HN016 to tilapia by IP and oral gavage challenge.

Infection route	Infection dose (CFU/fish)	Mortality rate (No.D/No.T[b])			
		HN016	YM001	△2 mutant	control
IP[a]	1.0×10^9	100% (40/40)	0.00% (0/40)	0.00% (0/40)	0.00% (0/40)
IP	0.5×10^9	100% (40/40)	0.00% (0/40)	0.00% (0/40)	
IP	1.0×10^8	90.00% (36/40)	0.00% (0/40)	0.00% (0/40)	
IP	1.0×10^7	92.50% (37/40)	0.00% (0/40)	0.00% (0/40)	
IP	1.0×10^6	82.50% (33/40)	0.00% (0/40)	0.00% (0/40)	
IP	1.0×10^5	57.50% (23/40)	0.00% (0/40)	0.00% (0/40)	
IP	1.0×10^4	37.50% (15/40)	0.00% (0/40)	0.00% (0/40)	
IP	1.0×10^3	10.00% (4/40)	0.00% (0/40)	0.00% (0/40)	
Oral gavage	1.0×10^{10}	95% (38/40)	0.00% (0/40)	0.00% (0/40)	
Oral gavage	1.0×10^9	92.50% (37/40)	0.00% (0/40)	0.00% (0/40)	

[a] Intraperitoneal injection.
[b] Nunber dead/number total.

fish) by oral gavage were 92.5% and 95%,respectively. However, fishes infected with YM001 or △2 mutant by either IP injection or oral gavage showed no mortality or signs of disease onset, which was similar as the control group.

Among all the fishes exposed to the △2 mutant vaccine through IP injection, no mortality, signs of disease, or adverse behavior was observed, and there was no dead fish in the back-passage safety studies. △2 mutant strain can be isolated from fishes of tank NO.1 to NO.50.

For tilapia immunized with △2 mutant by IP injection, the corresponding RPSs were 96.11% and 93.05% at 15 days and 30 days, respectively. The RPSs of the injection group at 15 and 30 days were essentially close, indicating that △2 mutant had good immunogenicity (Table 2).

3.4. Overviews of RNA transcriptomic and quantitative proteomic analyses profiles

In RNA-seq transcriptomic analyses, a total of 46,951,468 raw reads (11,020,366 and 13,441,342 for YM001; 13,323,472 and 7,790440 for △2 mutant, as well as 12,191,814 and 10,297,946 for HN016, respectively) were generated, and 67,297,690 clean reads were obtained after cleaning and quality checks. Approximately 97.89% (95.45–99.59%) of the mapped reads were acquired from the RNA-seq experiment, of which 97.06% (94.61–98.83%) were mapped to unique genomic locations (Fig. S1). A total of 825 genes (409 up-regulated and 416 down-regulated) and 16 proteins (13 up-regulated and 3 down-regulated) were significantly altered ($P < 0.05$) in △2 mutant vs HN016 group. Comparative transcriptomic and proteomic analyses identified 11 proteins (Fig. 3B and C) that had similar alteration trends at both mRNA and protein levels.

3.5. Bioinformatics analysis of differential expressed genes (DEGs) and differential expressed proteins (DEPs)

GO, an international standardized gene functional classification system, was used to classify the function of the differentially expressed mRNA and proteins of the bacterial strains. All mRNA and proteins with similar alteration trends were classified into three main categories: biological process, cellular component, and molecular function. The 11 DEGs (DEPs) were mainly belonged to single-organism process (Fig. 3A).

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database for systematic analysis of gene function and genomic informations. The 11 DEGs (DEPs) were associated with arginine biosynthesis, salivary secretion and pentose phosphate pathway. etc (Fig. 3A),which were related to energy metabolism and immunity (Figs. 2C and 4B).

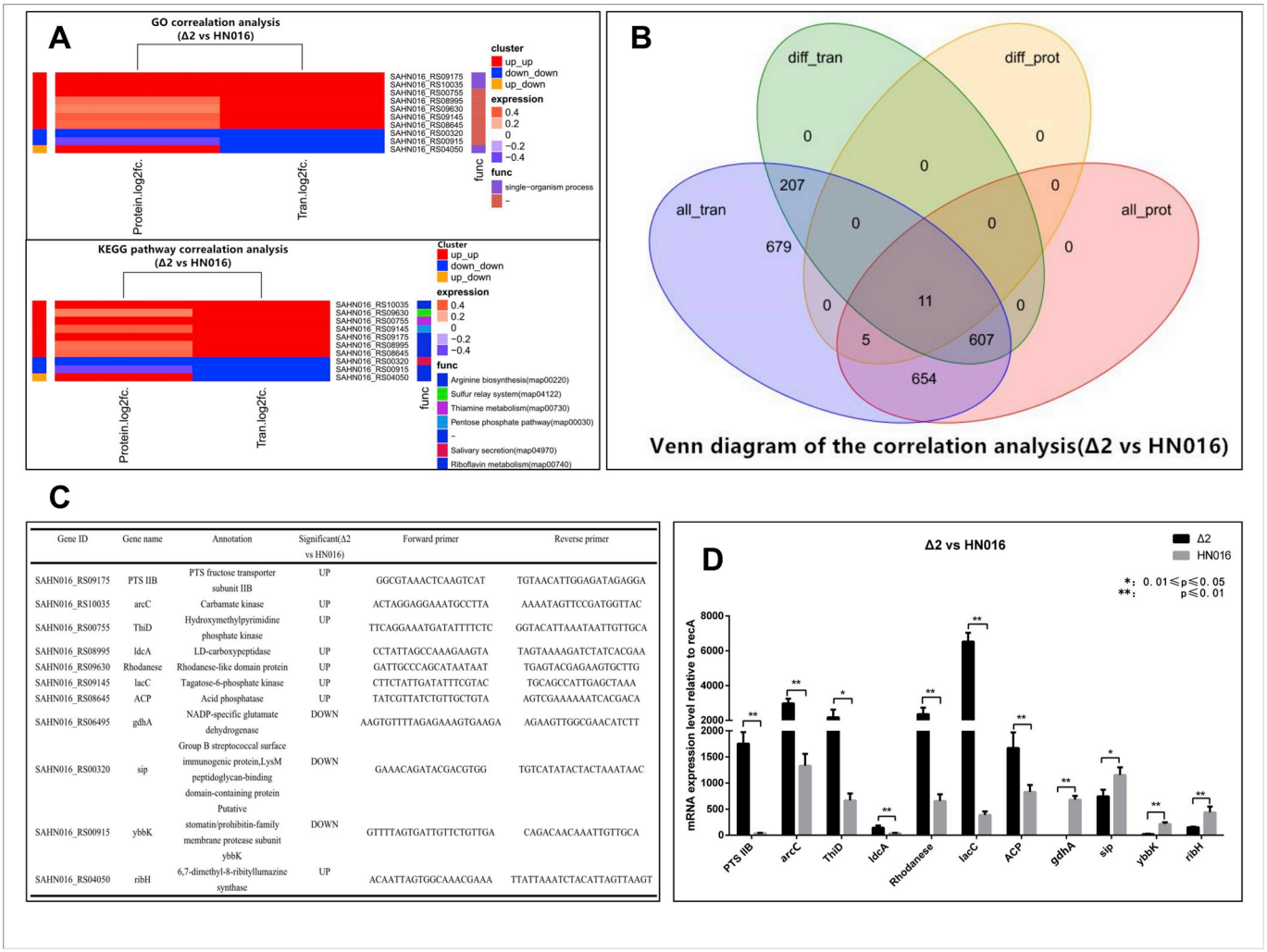
3.6. Histopathological analysis

Histopathological examination showed that there were severe lesions in the examined tissues of tilapia infected by HN016(Fig. 4A). For the brain tissue, the changes included edema, loose and thickening in meninges, disperse from brain matrix, interstitial inflammatory cell infiltration, capillary congestion, bleeding, large number of visible blue dye-stained Streptococcus particles. In the liver tissue, the lesions included congestion of central vein and hepatic sinus, vascular wall damage, endothelial cell necrosis and shedding, the inflammatory cell infiltration around central vein and pancreas, as well as the stained Streptococcus particles in the pancreas and liver sinus. Other changes including liver cells degeneration, necrosis,and disintegration were also observed. The changes from spleen included serious disorder of tissue structure, red blood cell infiltration in white pulp area, disappearance of lymphoid tissue structure, lymphocytes necrosis and number reduction, a large number of blue-stained Streptococcus granules in necrotic area, and scattered hemosiderin deposition. In the head kidney tissue, a lot of visible blue dye-stained Streptococcus particles were observed.

Table 2
The immune-protection of △2 mutant administered through IP injection in tilapia.

Treatment	Vaccination dose	Challenge dose	Challenge time	No.dead/ No.total[P]	Mean mortality ± S.D.[w]	Relative percent survival(RPS) ± S.D.	Challenge time	No.dead/ No.total[P]	Mean mortality ± S.D.[w]	Relative percent survival(RPS) ± S.D.
Injection	1.0×10^8	1.0×10^6	15 d	3/100	3.00 ± 3.83	96.11 ± 4.84	30 d	5/100	5.00 ± 5.03	93.05 ± 6.99
Oral	1.0×10^8	1.0×10^6	15 d	17/100	17.00 ± 2.00	74.80 ± 3.40	30 d	30/100	30.00 ± 5.16	53.16 ± 6.86
Blank control	–	1.0×10^6	15 d	70/100	70 ± 8.33	–	30 d	68/100	68 ± 5.66	–

[P] Total is represented by four replicate tanks of 25 fish each. Fish were challenged 15 and 30 days post-immunization by i.p. injection with 1×10^6 CFU/fish of *S.agalactiae* HN016 strain and monitored for 15 days post-challenge.
[w] Means analyzed by one-way analysis of variance using the GLM procedure and Duncan's multiple range test to determine significance at $P < 0.05$ (SPSS 19.0).



Gene ID	Gene name	Annotation	Significant(Δ2 vs HN016)	Forward primer	Reverse primer
SAHN016_RS09175	PTS IIB	PTS fructose transporter subunit IIB	UP	GGCGTAAACTCAAGTCAT	TGTAACATTGGAGATAGAGGA
SAHN016_RS10035	arcC	Carbamate kinase	UP	ACTAGGAGGAAATGCCTTA	AAAATAGTTCCGATGGTTAC
SAHN016_RS00755	ThiD	Hydroxymethylpyrimidine phosphate kinase	UP	TTCAGGAAATGATATTTTCTC	GGTACATTAAATAATTGTTGCA
SAHN016_RS08995	ldcA	LD-carboxypeptidase	UP	CCTATTAGCCAAAGAAGTA	TAGTAAAAGATCTATCACGAA
SAHN016_RS09630	Rhodanese	Rhodanese-like domain protein	UP	GATTGCCCAGCATAATAAT	TGAGTACGAGAAGTGCTTG
SAHN016_RS09145	lacC	Tagatose-6-phosphate kinase	UP	CTTCTATTGATATTTCGTAC	TGCAGCCATTGAGCTAAA
SAHN016_RS08645	ACP	Acid phosphatase	UP	TATCGTTATCTGTTGCTGTA	AGTCGAAAAAATCACGACA
SAHN016_RS06495	gdhA	NADP-specific glutamate dehydrogenase	DOWN	AAGTGTTTTAGAGAAAGTGAAGA	AGAAGTTGGCGAACATCTT
SAHN016_RS00320	sip	Group B streptococcal surface immunogenic protein,LysM peptidoglycan-binding domain-containing protein	DOWN	GAAACAGATACGACGTGG	TGTCATATACTACTAAATAAC
SAHN016_RS00915	ybbK	Putative stomatin/prohibitin-family membrane protease subunit ybbK	DOWN	GTTTTAGTGATTGTTCTGTTGA	CAGACAACAAATTGTTGCA
SAHN016_RS04050	ribH	6,7-dimethyl-8-ribityllumazine synthase	UP	ACAATTAGTGGCAAACGAAA	TTATTAAATCTACATTAGTTAAGT

Fig. 3. Significant differential expressed genes (proteins) between Δ2 mutant and HN016. A and B showes the omics analysis of Δ2 mutant and HN016. C showes the details of the DEGs. D showes the RT-qPCR verification result.

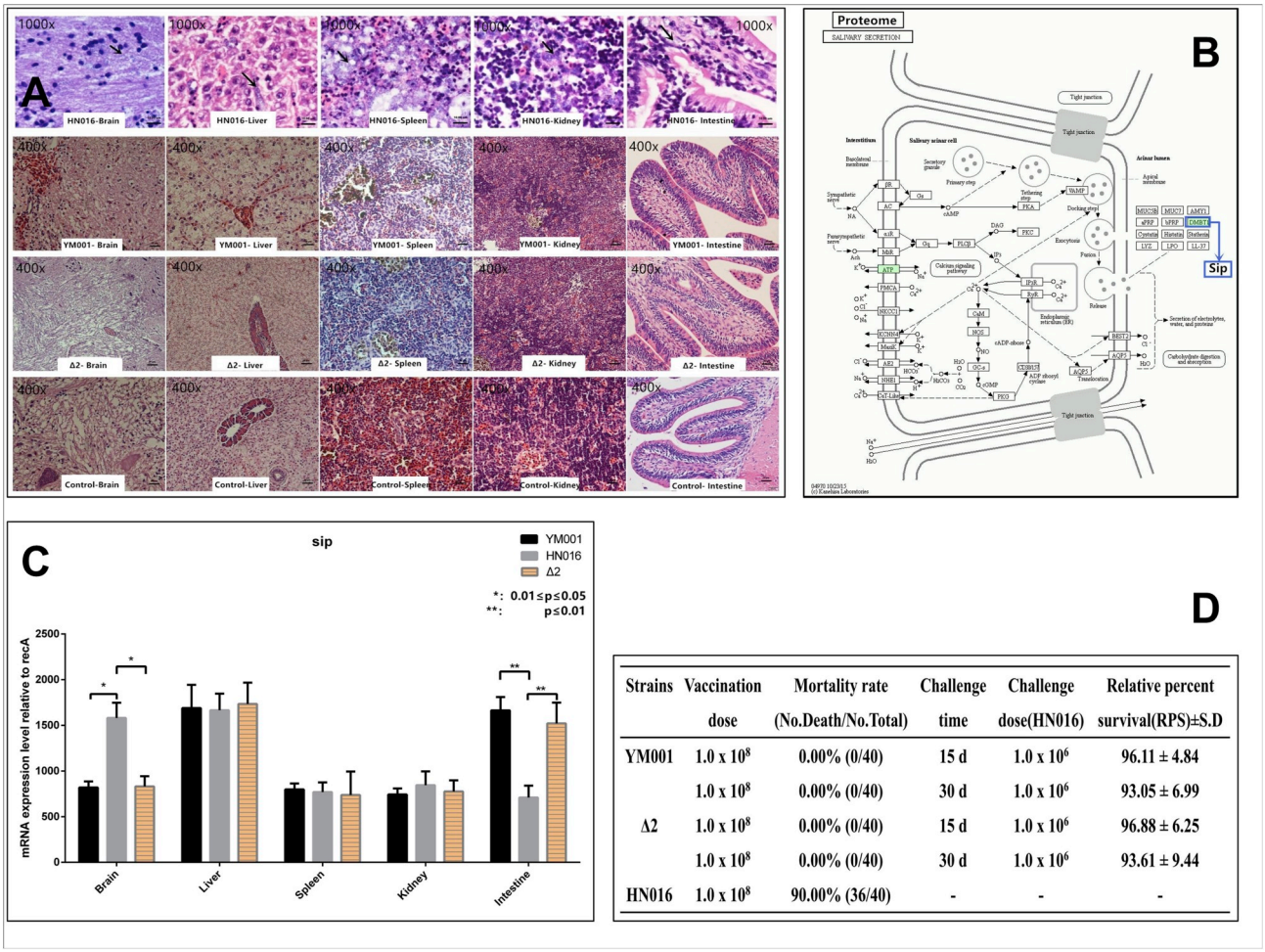
Intestinal serosal boundary was blurred, and visible blue dye-stained Streptococcus ranules were observed in the serosa, myometrium and submucosa. In contrast, no obvious histopathological changes were observed in fish injected with YM001, △2 mutant and control group. In general, the histopathological results demonstrated the different virulence characteristics of HN016, YM001 and △2 mutant.

3.7. RT-qPCR validation

The expression of related candidate genes in *S. agalactiae* strain and different tissues of tilapia that infected by *S. agalactiae* strains was verified by RT-qPCR. In Fig. 2D, we verified the expression levels of candidate genes in nitrogen metabolism and arginine biosynthesis signaling pathways. The results showed that the expression of these genes was significantly different in HN016, YM001 and △2 mutants; Fig. 3D showed the expression levels of 11 genes that showed significant different in protein levels in HN016 and △2 mutants, which further verified the reliability of the omics results. Fig. 4C showed the expression levels of gene encoding SIP protein in different tissues of tilapia infected with HN016, YM001, and △2 mutant strains, respectively. It was shown that the expression of sip gene in brain tissue of tilapia infected with HN016 was significantly higher than that in YM001 and △2-infected tilapia, while sip gene in the intestinal tissues was significantly highly expressed in tilapia infected with Δ2 mutant and YM001 than that in HN016 group.

4. Discussion

Previous studies have shown that the main difference between HN016 and its attenuated strain YM001 is the deletion of two large fragments (D1, D2). We speculated that the D2 fragment was the major functional fragment that regulated the virulence of the strain, thus we constructed a D2 fragment deletion strain Δ2 mutant by homologous recombination. The virulence experiments showed that Δ2 mutant completely lost the virulence to tilapia and had good safety. The backpassage safety experiment showed that the Δ2 mutant with 50 generations of continuous passage still had no virulence recovery and had good stability. Our backpassage safety assay is still undergoing, but it takes time to further verify. The RPSs of fishes immunized with Δ2 mutant (1.0×10^8 CFU/fish, one time) via injection and oral administration were 96.11 and 74.80% at 15 days, respectively, and 93.5% and 53.16% at 30 days, respectively. Compared with studies that Pridgeon et al., obtained attenuated strains by sparfloxacin screening [16] and Huang et al., obtained *S. agalactiae* recombinant DNA oral vaccine using attenuated *Salmonella typhimurium* as vector [17], Δ2 mutant not only can provide high level of immune protection, but also can be immunized by oral route, and RPS still up to 53.16% at 30 days, thus shows good immunogenicity. The attenuated oral vaccine strain YM001 that we prepared previously showed 93.61% and 53.12% of RPS after IP injection and oral immunization at 30 days, respectively, which was similar with Δ2 mutant vaccine, however, the growth rate of Δ2 strain



Strains	Vaccination dose	Mortality rate (No.Death/No.Total)	Challenge time	Challenge dose(HN016)	Relative percent survival(RPS)±S.D
YM001	1.0 x 10^8	0.00% (0/40)	15 d	1.0 x 10^6	96.11 ± 4.84
	1.0 x 10^8	0.00% (0/40)	30 d	1.0 x 10^6	93.05 ± 6.99
Δ2	1.0 x 10^8	0.00% (0/40)	15 d	1.0 x 10^6	96.88 ± 6.25
	1.0 x 10^8	0.00% (0/40)	30 d	1.0 x 10^6	93.61 ± 9.44
HN016	1.0 x 10^8	90.00% (36/40)	-	-	-

Fig. 4. Histopathological analysis and Sip protein. A shows the histopathological analysis results. B shows the omics analysis shows the omics analysis. C shows shows the RT-qPCR verification result. D shows the relative percent survival of the HN016, YM001 and Δ2 mutant.

was significantly enhanced. When cultured for 8 h under the same conditions, the bacterial density of Δ2 mutant grown in 500 mL modified Martin medium was about 5.81 × 10^9 CFU/ml, which was similar to wild strain HN018 (6.41 × 10^9 CFU/ml), while YM001 was about 1.58 × 10^9 CFU/ml. Thus, Δ2 mutant compensates for the low growth defect of YM001. On the other hand, previous studies have shown that HN016 can protect most of type Ia *S. agalactiae* infections in China [8], thus Δ2 mutant may also has a wide range of protection. Δ2 mutant has good safety, stability, immunogenicity and fast growth rate, and can provide high level of immune protection to tilapia. Thus, it is an excellent attenuated oral vaccine candidate strain.

Successful proliferation in the host environment is a prerequisite for pathogenic bacteria-induced diseases. Pathogens need to adapt to environmental factors such as strong acid, hypertonicity and oxidative stress [33], and evade the killing effect of host antibacterial molecules. Therefore, unlike the traditional definition of virulence factors, which are factors that directly affect bacterial virulence, factors affecting the growth of pathogenic bacteria can also be considered as virulence factors. Bacterial proliferation requires sufficient energy. Arginine metabolism is one of the important ways for bacteria to obtain energy. The bacteria convert arginine into ornithine through the arginine deiminase system (ADS), which simultaneously produces ammonia and energy [34]. The produced ammonia can raise the pH of the environment and allow the bacteria to survive in a deadly acidic environment [35]. Multi-omics analysis showed that the expression of important enzymes in the arginine pathway, including Carbamate kinase (CK), NADP-specific glutamate dehydrogenase (NADP-GDH) and Glutamine synthetase type I (glnA), were significantly different in three strains. CK was one of the three constituent enzymes of ADS [36]. Because the expression of CK in Δ2 mutant and HN016 was significantly higher than that in YM001, more energy can be obtained for proliferation through the ADS pathway, thus the growth of Δ2 mutant and HN016 was significantly better than that of YM001.NADP-GDH catalyzes the reversible oxidative deamination of glutamate to alpha-ketoglutarate and ammonia [37]. Glutamine synthetase is involved in nitrogen metabolism via ammonium assimilation, and catalyzes the ATP-dependent biosynthesis of glutamine from glutamate and ammonia [38]. Tullius et al. constructed a *glnA* mutant of *M. tuberculosis* by allelic exchange and found that the mutant was deficient for growth in human THP-1 macrophages, while complement of *glnA* fully restored the virulence of mutant, indicating that *glnA* was closely related to the virulence of *M. tuberculosis* [39]. It has been shown that *S. agalactiae* can survive for a long time after phagocytosed by phagocytic cells such as macrophages and neutrophils [40–42], thereby escaping the killing effect of active antibacterial molecules in the blood, which is the basis of *S. agalactiae*-induced bacteremia and subsequent meningitis [43]. Down-regulation of *glnA* and non-expression of NADP-GDH gene resulted in the decreased viability of YM001 in macrophages, causing the inability of bacteria to escape the attack of antibacterial molecules and reduced viability. Thus, CK, NADP-GDH and Glutamine synthetase type I are important factors influencing the virulence of Δ2 mutant.

Sip is a surface immunogenic protein (Sip) localized on the bacterial surface. It is an important adhesion and colonization factor of bacteria [44] and has high homology in human, bovine and fish [45]. Vidová

et al. found that the Sip protein with a deletion of conserved LysM domain showed significantly reduced immunogenic protection in mice [46]. Brodeur et al. and Li et al. isolated the whole Sip of GBS isolated from humans and bovines was expressed successfully in BL21(DE3) transformed with pET32a-sip, but when the BL21 (DE3) host was used in conjunction with pET vectors, some toxic proteins could not be successfully reproduced [47,48]. He et al. found that Sip protein can not be expressed in BL21 or Rosetta strains, but the Sip without N-terminal signal peptide and LysM domain was successfully over-expressed, suggesting that N-terminal signal peptide and LysM may contribute to the Sip toxicity [49]. In the present study, the expression of Sip with LysM domain was significantly decreased after deletion of D2 fragment. However, RT-qPCR quantitative analysis showed that there was no significant difference for the expression of *sip* gene in liver, spleen, and kidney tissues of tilapia infected with YM001, HN016 or Δ2 mutant. The expression level of *sip* in brain tissue of fish infected with HN016 was significantly higher than that in YM001 group and Δ2 mutant group, which was positively correlated with the virulence. A typical symptom of *S. agalactiae* disease is that bacteria can break through the blood-brain barrier (BBB) and reach the brain, causing meningitis. The expression levels of *sip* in intestinal tissues of fish infected with YM001 or Δ2 mutant were significantly higher than that in the HN016-infected group, which was positively correlated with the immunogenicity of the strains. It is possible that the intestines of fish, especially the posterior segment, are immunologically active and have a variety of immune cells, including B cells, macrophages, granulocytes and T cells [50,51]. YM001 and Δ2 mutant may interact with immune cells and induce the body to produce long-lasting immune protection, while HN016 can survive in macrophages, invade other organs with blood circulation, and cause acute bacteremia and other symptoms. Most of fish die quickly after infection, thus the expression level of Sip in the intestine was low. The specific mechanism still needs further research.

5. Conclusion

In this study, we constructed a D2 fragment knockout strain of HN016 and verified that deletion of D2 fragment caused the loss of HN016 pathogenicity to tilapia, and explored the factors affecting the growth and virulence of the strain. We also demonstrated that Δ2 mutant is a safe, stable, immunogenic and fast-growing tilapia *S. agalactiae* attenuated vaccine strain.

Ethics statement

Animal experiments were conducted in strict accordance with the Chinese animal experiment ethical inspection, under project licence number: GXU2015039, approved by the Guangxi University, CHINA.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.081.

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