Functional Analysis of Fis in Aeromonas Veronii TH0426 Reveals a Key Role in the Regulation of Virulence and Adhesion

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Abstract

Background: *Aeromonas veronii* is a comorbid pathogen that can infect humans, animals, and various aquatic animals. In recent years, an increasing number of cases of *A. veronii* infection have been reported, and its virulence and drug resistance have also been increasing gradually, indicating serious risks. This bacterium not only threatens public health and safety but also causes considerable economic loss in the aquaculture industry; however, little is known about its pathogenic mechanism.

Results: In this study, we first constructed the *A. veronii* TH0426 *Fis* gene deletion strain Δ*Fis* and the complementation strain C-*Fis* through homologous recombination technology. The results showed that the adhesion and invasion ability of the Δ*Fis* deletion strain towards Epithelioma papulosum cyprini (EPC) cells and the cytotoxicity were 3.8-fold and 1.3-fold lower, respectively, than those of the wild-type strain. The animal pathogenicity of Δ*Fis* was 2.9-fold lower than that of the wild-type strain. In addition, the bacterial load of the deletion strain Δ*Fis* in crucian carp was significantly lower than that of the wild-type strain, and the load decreased with time. In summary, deletion of the *Fis* gene led to a decrease in the virulence of *A. veroni*i.

Conclusions: Our research results show that the deletion of the *Fis* gene significantly reduces the virulence and adhesion ability of *A. veronii* TH0426. Therefore, the *Fis* gene plays a vital role in the pathogenesis of TH0426. This preliminary study of the function of the *Fis* gene in *A. veronii* will help researchers further understand the pathogenic mechanism of *A. veronii*.

1. Background

A.veronii is a gram-negative facultative anaerobic bacterium that is widely found in freshwater, seawater, silt, and soil[1, 2]. It was isolated from patients with diarrhoea and wound infection by the Centers for Disease Control and Prevention (CDC) in 1983. It is a zoonotic pathogen that can cause disease in humans, animals, and a variety of aquatic organisms[3, 4]. In recent years, there have been an increasing number of cases of *A. veronii* infecting various aquatic animals, warm-blooded animals, and even humans around the world[5-10]. *A. veronii* can cause various diseases, such as skin rot disease, gill rot, and sepsis, and even morbidity and death in aquatic animals. Human infection can cause gastroenteritis, pneumonia, and traumatic infections, and these occurrences not only cause considerable economic loss in the breeding industry but also threaten food quality and public health[11-13]. At present, research on *A. veronii* is limited to the isolation and identification of certain diseased animals, and there is very little research on its pathogenic mechanism[14-17]. Therefore, investigation of its virulence factors will enable us to better understand its pathogenic mechanism, which will contribute to the prevention and control of the occurrence of large-scale infection by *A. veronii.*

Almost every major process in cells is carried out by macromolecular machines[18]. Protein complexes have highly coordinated moving parts, and the conformational changes of these moving parts are driven by energy. These macromolecular machines require additional equipment that plays a role in their

assembly. This supplementary device, usually referred to as a molecular chaperone protein, can help in the non-covalent assembly of other proteins or protein complexes[19, 20]. The remodelling function of molecular chaperones can make the substrate protein more proteolytic. This function serves as a quality control mechanism to eliminate faulty components that cannot be integrated correctly. Chaperones can also modulate the activity of protein complexes by mediating the degradation or availability of specific components[21, 22]. Evolution-related molecular chaperones that play a role in the assembly or regulation of molecular machines may be related to a variety of cellular activities. This is the case with members of the AAA^+ family, which represent ATPases involved in diverse cellular activities[23, 24]. The AAA^+ family of ATPases is present in all living organisms, and these proteins are involved in various cellular processes, including membrane fusion, proteolysis, and DNA replication[25]. Many AAA^+ family proteins usually perform chaperone-like functions, such as promoting protein folding and unfolding, protein complex assembly or disassembly, and protein transport and degradation, but some other proteins play a role in replication, recombination, repair, and transcription[26, 27]. The *Fis* gene encodes an ATPase of the AAA^+ family, so this gene may affect protein folding, which is related to the pathogenicity and virulence of bacteria. However, there is no research showing the function of the *Fis* gene in *A. veronii*, so we will explore this aspect to understand the infection mechanism of *A. veronii.*

In this study, we deleted the main sequence of the *Fis* gene in *A. veronii* through homologous recombination and constructed a *ΔFis* mutant strain and an overexpression strain, C-*Fis*. We compared and analysed the growth characteristics, haemolytic activity, biofilm formation ability, pathogenic ability, adhesion ability, and cytotoxicity of the three strains. Our findings will further clarify the pathogenic mechanism of the bacterium and provide a deeper theoretical basis. The results will allow us to reduce the harm caused by *A. veronii* to public health and lay a theoretical foundation for the development of vaccines for bacteria.

2. Results

2.1 Construction and detection of Δ*Fis* and C-*Fis*

To determine whether the Δ*Fis* and C-*Fis* strains were successfully constructed, we extracted Ribonucleic Acid (RNA) from the deletion strain Δ*Fis* and the wild-type strain TH0426 using reverse-transcribed Complementary Deoxyribonucleic acid (cDNA) as the template for verification with the primers $P_{3\text{-}1}/P_{3\text{-}2}$. The target fragment could not be amplified from the deletion strain Δ*Fis*, but a 1409 bp fragment was amplified from the wild-type strain, which proved that the results were consistent with expectations. The RNA of the complementation strain C-*Fis* was extracted for PCR verification, and a 1409 bp fragment was amplified, which was the same as the result for the wild-type strain. In contrast to the wild-type strain, Δ*Fis* could not express the *Fis* gene, while the *Fis* gene expression level of C-*Fis* was 223-fold that of the wild-type strain, indicating that Δ*Fis* and C-*Fis* were successfully constructed.

2.2 No significant effect of Δ*Fis* on growth

After culturing the deletion strain Δ*Fis* and the complementation strain C-*Fis* on LB solid medium, the colony morphology was observed and compared with that of the wild-type strain TH0426, but there was no significant difference. The three strains were all gram-negative bacteria that had short and rod-shaped cells that existed alone or in pairs. The colony shape was round, the surface was smooth, and the edges were neat. The growth rate of the three strains was determined to clarify the function of the *Fis* gene during the growth process. The results showed that the growth ability of the deletion strain Δ*Fis* was not significantly different from that of the complementation strain C-*Fis* and the wild-type strain TH0426 (Fig 1).

2.3 Haemolytic activity

Comparison of the haemolytic activity of the deletion strain Δ*Fis*, the complementation strain C-*Fis* and the wild-type strain TH0426 showed that the haemolytic activity did not change significantly among the three strains, indicating that the *Fis* gene has no significant relationship with the haemolytic activity of the strain.

2.4 Effect of *Fis* gene deletion on motility

Previous research by this research group showed that TH0426 has the ability to swim. The swimming abilities of the deletion strain Δ*Fis* and the complementation strain C-*Fis* were tested. The results showed that the swimming distance of Δ*Fis* was 1.65 ± 0.35 cm, the swimming distance of C-*Fis* was 1.65 ± 0.40 cm and the swimming distance of TH0426 was 1.35 ± 0.15 cm (Fig 2), so there was no significant difference in swimming ability among Δ*Fis*, C-*Fis*, and TH0426.

2.5 Effect of Δ*Fis* gene on biofilm formation ability

The OD_{575} value can reflect the amount of biofilm formed by a strain and the intensity of biofilm formation. The results show that the OD_{575} values of Δ*Fis* and C-*Fis* were 1.27 ± 0.23 and 1.23 ± 0.38, respectively, while the OD_{575} values of the wild-type strain TH0426 and negative control PBS were 1.74 ± 0.59 and 0.20 ± 0.02 (Fig 3), respectively. This indicates that due to inactivation of the *Fis* gene, the biofilm formation ability of the Δ*Fis* deletion strain was reduced, but the difference was not significant. Therefore, the *Fis* gene cannot regulate the biofilm formation of *A. veronii.*

2.6 *Fis* deletion reduced the virulence of TH0426

The toxicity of the deletion strain Δ*Fis*, the complementation strain C-*Fis* and the wild-type strain *A. veronii* TH0426 was tested by the cytotoxic kit. The results showed that the cell viability of EPC cells after inoculation with the deletion strain Δ*Fis* was 1.38-fold that of the wild-type strain *A. veronii* TH0426 (P <

0.001) (Fig 4). Since cell viability is directly proportional to the number of viable cells, this finding indicates that the deletion strain Δ*Fis* was less cytotoxic than the wild-type strain *A. veronii* TH0426, while the complementation strain C-*Fis* was more cytotoxic than the deletion strain Δ*Fis.*

2.7 Adhesion and invasion ability detection

The test results for the adhesion and invasion abilities of the EPC cells showed that the bacterial adsorption rate of TH0426 was 3.8-fold that of Δ*Fis* (Fig 5). The adhesion and invasion abilities of the deletion strain Δ*Fis* were significantly weaker than those of the wild-type strain *A. veronii* TH0426, while the adhesion and invasion abilities of the complementation strain C-*Fis* were restored but not obvious. This indicates that the *Fis* gene has a certain influence on the adhesion and invasion abilities of *A. veronii*.

2.8 Determination of the half-lethal dose (LD_{50}) n zebrafish

A zebrafish infection model was established and used to calculate the zebrafish median lethal dose to determine the change in virulence of the deleted strain Δ*Fis*. The LD_{50} value of TH0426 was (7.60 ± 0.32) × 10^5 CFU/tail, the LD_{50} value of the deletion strain Δ*Fis* was (2.28 ± 0.16) × 10^6 CFU/tail, and the LD_{50} value of the complementation strain C-*Fis* was (1.37 ± 0.29) × 10^6 CFU/tail. The results showed that the LD_{50} value of Δ*Fis* was 2.99-fold higher than that of TH0426 ($P < 0.001$), indicating that the virulence of Δ*Fis* was lower than that of the wild type (Fig 6) and that C-*Fis* exhibited slight virulence recovery.

2.9 Detection of virulence gene expression

The detection results for the four virulence genes aerolysin (*aer*), serine protease (*ser*), elastase (*ahyB*) and lipase (*lip*) in the wild-type strain, deletion strain and complementation strain are shown in Fig 7. The serine protease gene expression level in the deletion strain Δ*Fis* was 2.96-fold that in TH0426, and the elastase gene expression level in the deletion strain Δ*Fis* was 0.38-fold that in TH0426.

2.10 Bacterial load test

The wild-type strain, deletion strain and complementation strain were used to challenge crucian carp. The results showed that the average bacterial loads in the TH0426 wild-type strain in the liver, spleen, and kidney after 24 h were 5.41 × 10^5 CFU/g and 4.63 × 10^5 CFU/g, and 2.18 ×10^4 CFU/g, respectively; the loads in the Δ*Fis* deletion strain were 1.44 × 10^5 CFU/g, 3.57 × 10^3 CFU/g and 4 × 10^3 CFU/g, respectively.

Seventy-two hours after the crucian carp were challenged, the average bacterial loads in the TH0426 wild-type strain in the liver, spleen and kidney were 6.60 × 10^2 CFU/g, 1.95 × 10^2 CFU/g, and 1.56 × 10^3 CFU/g,

respectively; the loads in the deletion strain Δ*Fis* were 85 CFU/g, 103 CFU/g, and 295 CFU/g, respectively. The experimental results showed that at 24 h and 72 h, the bacterial load of Δ*Fis* was significantly lower than that of the wild-type strain, and the *Fis* gene affected the pathogenicity of TH0426.

3. Discussion

In recent years, the number of research reports on *A. veronii* has gradually increased, and most of the reported illnesses are relatively serious, which has aroused much attention. *A. veronii* can produce a large number of virulence factors, such as haemolysin, enterotoxin, and adhesion factors[28]. These virulence factors play a major role in aquatic animal, livestock and even human infections. *A. veronii,* a gram-negative bacterium, contains a variety of secretion systems, among which type III and type VI secretion systems are related to the virulence of most gram-negative bacteria [29, 30]. Based on careful multiple-sequence alignments, Neuwald et al. [31] proposed a broader AAA^+ family including the classic AAA family in 1999 and later discovered that AAA^+ family proteins usually perform chaperone-like functions and help in the assembly, manipulation, or disassembly of protein complexes. Teru et al. explored the structure of AAA^+ family proteins and found that the central ATPase domain (called the AAA^+ module) of approximately 250 amino acids is structurally conserved[32]. Nahar et al.[33] found that the AAA family protein Cdc48-Ubx2 complex regulates the conversion of the outer mitochondrial membrane protein Fzo1 by regulating the ubiquitination state of the substrate. Li et al.[34] found that AAA^+ ATPase TER94 can interact with the baculovirus early protein LEF3 and helicase to transport and further recruit proteins related to viral replication to establish a viral replication factory. Khong et al.[35] found that loss of the AAA family ATPase p97/VCP leads to a decrease in the actin dynamics of knockdown cells, impaired cell motility, and increased RhoA protein levels, which participate in the proteasome-dependent protein degradation pathway. The *Fis* gene encodes an ATPase of the AAA^+ family in the type VI secretion system, and other members of the family participate in a variety of cellular processes. Therefore, the study of the *Fis* gene is of great significance to further explain the pathogenic mechanism of *A. veronii*.

In this study, the biological characteristics of the *A. veronii* wild-type strain TH0426, deletion strain Δ*Fis* and complementation strain C-*Fis* were analysed, and it was found that after gene deletion, the growth ability of the bacteria was almost unchanged compared with that of the wild-type strain. The swimming test showed that the swimming and surging distances of Δ*Fis* were almost unchanged compared with those of the wild-type strain. Analysis of the *Fis* gene showed that it is not involved in the proliferation and motility of TH0426 cells. This result is similar to the results of Tian et al. [36] for the acid bacteriophage xj12, in which deletion of the type VI secretion system AAA ATPase gene *clp B* did not affect the proliferation and motility of the strain. However, this finding is different from that of Li Jing et al.'s[37] study of the deletion of the *moxR* gene related to the acid phagocytosis Aac-5AAA ATPases of watermelon, which caused the bacterium's motility and growth ability to decrease. We speculated that this trait may be affected by nucleotide metabolism. The cause remains unknown, and further research is needed.

Bacterial biofilms are aggregate film-like substances formed by bacteria to envelop themselves to adapt to the environment. Biofilms confer drug resistance and immune tolerance to bacteria, leading to difficulties in disease treatment. The biofilm formation ability test showed that Δ*Fis* exhibited almost no change compared with the wild-type strain. This result is similar to the result of Tian et al.[36], who studied the xj12 strain and found that deletion of the *clpB* gene did not affect the biofilm formation ability of the strain. However, the finding is distinct from that of Li Jing et al.'s[37] study, in which deletion of the *moxR* gene related to the Aac-5-mediated acid phagocytosis of watermelon resulted in enhancement of the biofilm ability of the strain. It is speculated that this trait is related to the different genes, and the *Fis* gene does not participate in the formation of TH0426 biofilms.

For the process of bacterial infection, recognition of the receptors on the host's surface is essential. After some biological reactions, the bacteria invade the host. Virulence factors can promote this process. In the cell adhesion and invasion tests, Δ*Fis* exhibited reduced cell adhesion and invasion abilities compared with TH0426. It is speculated that deletion of the *Fis* gene reduces the secretion of toxins and adhesion of TH0426 and reduces the ability to recognize the surface receptor of the host. This suggests that the *Fis* gene affects the adhesion ability of bacteria. Δ*Fis* exhibited reduced toxicity towards EPC cells. It was speculated that the *Fis* gene affects the virulence of TH0426 towards host cells, which is similar to the result of our study. When Seemuller et al.[38] studied the AAA^+ ATPase AP460 protein of the Candidatus *Phytoplasma mali* strain, they found that it was related to the inhibition of virulence. The results of the zebrafish LD_{50} test showed that compared with that of the wild-type strain, the LD_{50} of Δ*Fis* towards zebrafish increased and the virulence decreased, which indicated that the *Fis* gene affected the pathogenicity of TH0426. The virulence gene expression test results showed that *Fis* deletion increased the expression of serine protease and reduced elastase expression in TH0426. It is speculated that the *Fis* gene is involved in the protein transcription process, resulting in *Fis* gene deletion reducing the ability of bacteria to recognize the surface receptor of the host. This resulted in reduced pathogenicity towards cells and zebrafish. This result is similar to the view of Neuwald et al.[31], who suggested that the AAA^+ family *Lon* and *Clp* proteins are involved in DNA replication, recombination, and restriction in *E. coli* and other bacteria. The bacterial load test results showed that the bacterial load of Δ*Fis* was significantly lower than that of the wild-type strain, which indicated that the *Fis* gene affected the pathogenicity of TH0426. It is speculated that deletion of the *Fis* gene reduces the cell adhesion ability of TH0426 cells, resulting in a decrease in animal pathogenicity.

In summary, we successfully constructed an *A. veronii* Δ*Fis*-deleted strain through homologous recombination technology, verified part of the biological function of the *Fis* gene in *A. veronii* through preliminary exploration, and found that lack of the *Fis* gene affected the adhesion ability of *A. veronii* and that the virulence and pathogenicity decreased significantly. This lays the foundation for the subsequent development of *A. veronii* attenuated vaccines and the exploration of pathogenic mechanisms.

4. Conclusions

In summary, we successfully constructed the *Fis* gene deletion strain and the complementation strain for the first time and characterized the biological function of this gene through the analysis of biological characteristics. The *A. veronii* TH0426 strain lacking the *Fis* gene exhibited no significant changes in biofilm formation ability, but the bacterial cell adhesion ability, cytotoxicity and animal pathogenicity were significantly reduced. These results indicate that the *Fis* gene plays a crucial role in the pathogenesis of *A. veronii*. This study explored the function of the *Fis* gene in *A. veronii* for the first time and provided references for further exploration of the pathogenicity of *A. veronii.*

5. Methods

5.1 Bacterial strains, plasmids, and growth conditions

The wild-type strain *A. veronii* TH0426 used in this study was initially isolated from a farmed *Pelteobagrus fulvidraco* in Zhejiang Province, China. The original strains and plasmids were stored by the Preventive Veterinary Research Laboratory of Jilin Agricultural University. The bacterial strains and plasmids used in this study are listed in Table 1. All the *A. veronii* strains were grown in Luria-Bertani (LB) medium and Rimler-Shotts (RS) selective medium at 30 °C. The host bacterium DH5α strains and engineered *E. coli* strain WM3064 used in this study were grown in LB broth or plated on LB agar plates. Three vectors, namely, pEASY-Blunt Zero (pEASY), the broad-host-range expression plasmid pBBR1-MCS, and the suicide plasmid pRE112, were utilized for gene expression. When required, appropriate antibiotics were added at the following final concentrations: ampicillin (Amp, 100 μg/ml) and chloramphenicol (Cm, 45 μg/ml).

Table 1

Bacterial strains and plasmids used in this study.

Strains or Plasmids	Description	Source or Reference
Strains		
A. veronii TH0426	Wild-type strain, Amp^r	This study
Δ*Fis*	Isogenic *Fis* mutant of strain TH0426	This study
C-*Fis*	Mutant Δ*Fis* complemented with intact *Fis* gene	This study
E. coli Trans1-T1	F-ϕ80(*lacZ*)ΔM15Δ*lacX*74hsdR(rk^-,*mk*+) Δ*recA*1398*end*A1*ton*A	TransGene Biotech
E. coli WM3064	*thrB1004 pro thi rpsL hsdS lacZΔM15RP4-1360(araBAD)567ΔdapA1341: :[erm pir(wt)]*	Stored in our lab
E. coli DH5α-λpir	*λpir* lysogen of DH5α	Stored in our lab
Plasmids		
pEASY-Blunt Zero	TA cloning vector, Amp^r	TransGene Biotech
pEASY-UD *Fis*	Carrying the flanking region of the ORF for *Fis* TA cloning, Amp^r	This study
pRE112	pGP704 suicide plasmid, *pir* depengent, *oriT*, *oriV*, *sacB*, Cm^r	Stored in our lab
pRE112-UD *Fis*	pRE112 carrying the flanking region of the *Fis* ORF, Cm^r	This study
pBBR1-MCS	Broad-host range vector, Cm^r	Stored in our lab
pBBR-*Fis*	pBBR carrying of 1681bp containing the promoter and *Fis* ORF, Cm^r	This study

5.2 Experimental Fish

We purchased healthy zebrafish weighing 0.5 ± 0.03 g and crucian carp weighing 300 ± 0.5 g from a fish farm (Changchun, Jilin, China). The crucian carp and zebrafish were fed the basal diet twice a day in the amount of 2% of their body weight for 2 weeks. Crucian carp were acclimatized in flow-through aquariums at 26 ± 1.0 ℃ and zebrafish were maintained in Racirculating Aquaculture Systems at 28 ± 1.0 ℃, with natural photoperiod. During the whole experiment periods, the physicochemical parameters of the water were measured daily (5.6 ± 0.45 mg/L of dissolved oxygen, 0.12 ± 0.01 mg/L of ammonia, 0.015 ± 0.003 mg/L of nitrate and 7.8 ± 0.5 of pH). After the study, all remaining experimental animals were euthanized by bringing the concentration of cove oil in the water to 80 mg/L. This study was conducted following the Jilin Agriculture University Institutional Animal Care and Use Committee

(JLAU08201409), and the experimental procedures were performed in compliance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023).

5.3 Construction of the *A. veronii* deletion and complementation strains

To construct the *Fis* gene deletion strain, partial deletion of the *Fis* gene was conducted by homologous recombination. Briefly, the genome of the TH0426 strain was used as a template to amplify the flanking region of the *Fis* gene with the primers $P_{1\text{-}1}/P_{1\text{-}2}$ and $P_{1\text{-}3}/P_{1\text{-}4}$, and the upstream (named S1) and downstream (named S2) regions of the target gene *Fis* were amplified by Polymerase Chain Reaction (PCR). Using S1 and S2 as templates, the primer pair $P_{1\text{-}1}/P_{1\text{-}4}$ was utilized to amplify the fragment lacking the *Fis* gene mutation, named M *Fis*. The two purified flanking regions were then ligated by fusion PCR and inserted into a linear vector, pRE112, digested at the same restriction site, and the construct obtained was named pRE112-M *Fis*. The correctly sequenced recombinant suicide plasmid pRE112-M *Fis* was transformed into competent *E. coli* WM3064 cells and then conjugated into the wild-type strain *A. veronii*. A plate containing Amp and Cm was used to screen the strains for the first conjugative transfer, and then, the second homologous recombination was induced on the plate containing Amp + 10% sucrose.

The *Fis* gene fragment carrying the promoter region (2,252 bp) was amplified and ligated into the broad-host-range expression plasmid pBBR1-MCS of gram-negative bacteria to construct the expression plasmid *Fis*. Finally, Reverse Time-Polymerase Chain Reaction (RT-PCR) was used to identify the mutant Δ*Fis* and the complementation strain C-*Fis*. All primers utilized in this study were listed in Table 2.

Table 2

Primers used in study

Target genes	Primers	Sequence (5'~3')
Fis upstream homology arm sequence	P_{1-1} (*Xba* I)	GCTCTAGACGATGCGCCGCTCGATC
	P_{1-2}	GACACTTGTGCGCGAGGGCGAATGCGA
Fis downstream homology arm sequence	P_{1-3}	CGCCCTCGCGCACAAGTGTCAGAAACTGGAGGT
	P_{1-4} (*Kpn* I)	GGGGTACCCCTTGTCAGGGCACTGGGCC
Fis ORF and its external sequence	P_{2-1}	TTGTCGCTGGGCCCGCTTCA
	P_{2-2}	CTGGCCGATCAGCAGGATGA
Fis ORF internal sequence	P_{3-1}	TCGCAACGGGATGAACAACA
	P_{3-2}	CGCAGGCGAGCTTCAATCA
Promoter sequence	P_{4-1} (*BamH* I)	CCGGATCCCTGTTTATGGGCGGC
	P_{4-2}	CTTGCTCCATGGGCGTGCTCT
Fis ORF sequence	P_{4-3}	CGCCCATGGAGCAAG
	P_{4-4} (*Hind* III)	CGAAGCTTTCAGTTCACCTCCAGTTTC
16S rRNA sequence (for real-time PCR)	P_{5-1}	GCCACGTCTCAAGGACACAG
	P_{5-2}	TGGGGAGCAAACAGGATTAGA
Fis ORF sequence (for real-time PCR)	P_{6-1}	CTCTACCACCGTCTC
	P_{6-2}	CAGTCCCTTGTCATC
pRE112 vector sequence	P_{7-1}	GCGATGAGTGGCAGGGC
	P_{7-2}	TTACCGACTGCGGCCTGAGT
pBBR1-MCS vector sequence	P_{8-1}	TAAGTTGGGTAACGCCAGG
	P_{8-2}	GAGTTAGCTCACTCATTAGGC

5.4 Comparison of colony morphology and determination of the growth curve

Δ*Fis*, C-*Fis,* and *A. veronii* TH0426 were inoculated into LB at 1% of the culture volume and cultured at 30 °C for 12 h. The concentrations of the three strains were adjusted to the same value by colony counting. The final concentration was adjusted to 1×10^6 CFU/mL, and the morphology was observed by Gram staining. Then, the same amount of bacterial liquid was dropped onto a solid LB plate, and the colony morphology was observed after incubation for 35 h at 30 °C. The experiment was replicated three times.

Δ*Fis*, C-*Fis*, and *A. veronii* TH0426 at the adjusted concentrations were inoculated into liquid LB medium at 1% of the culture volume and cultured at 30 °C and 170 rpm for 13 h. The OD_{600} was measured at intervals of 1 h and recorded. This experiment was replicated three times.

5.5 Determination of haemolytic activity

To evaluate whether the haemolysin activity of *Fis* was affected, we performed a haemolytic activity experiment. Briefly, according to a previously described method, the cell densities of Δ*Fis*, C-*Fis*, and TH0426 were adjusted to be equal. Equal volumes of the bacterial suspensions were inoculated onto a sheep blood agar plate, and the cells were cultured at 30 °C for 12 h. The experiment was repeated three times.

5.6 Motility detection

Previous research results from this research laboratory have shown that TH0426 has swimming ability. We evaluated the swimming motility of the bacteria by measuring the colony diameter of bacteria growing on a plate containing 0.5% agar and 5% glucose. The swimming ability of Δ*Fis*, C-*Fis*, and TH0426 was tested.

5.7 Biofilm assay

The biofilm detection procedure was based on the method of Müsken M et al. [39]with appropriate modifications. The concentrations of Δ*Fis*, C-*Fis*, and TH0426 were adjusted to 1×10^6 CFU/mL, and 180 μL of LB broth and 20 μL of bacterial solution were added to a 96-well plate; sterile Phosphate Buffered Saline (PBS) was added to the control wells. Ten repeat wells were set up for each strain. The 96-well plate was sealed with parafilm and incubated at 28 °C for 24 h. After the incubation was complete, the liquid in each well was aspirated, and the well was washed twice with sterile PBS. Then, 200 μL of 99% methanol was added, and the cells were fixed for 20 min. The methanol was then aspirated, and 200 μL of 0.1% crystal violet staining solution was added to each well. The solution was then aspirated, and the wells were washed three times with sterile PBS. Then, 200 μL of 33% acetic acid was added to each well,

and the plate was incubated for 5 min. Finally, the biofilm formation ability of each strain was determined by OD_{575} analysis, which was repeated three times[40].

5.8 Cytotoxicity analysis

A cytotoxic kit was used to detect the toxicity of the deletion strain Δ*Fis* and the wild-type strain TH0426 to EPC cells according to the manufacturer's instructions.

5.9 Bacterial adhesion to and invasion of EPC cells

The adhesion and invasion abilities of Δ*Fis*, C-*Fis*, and TH0426 to EPC cells were tested[41]. EPC cells were cultured in Medium 199 (M199) medium containing 10% foetal bovine serum and 1% dual antibodies (penicillin and streptomycin) at 25 °C in a 5% CO_2 incubator. In brief, EPC cells were subcultured and counted, and the cell density was adjusted. And the cells were then seeded on a 24-well cell culture plate. The medium was aspirated and discarded after culturing to a monolayer, and the cells were washed twice with M199 (without antibiotics and serum). Then, M199 was used to dilute the bacterial solution at a ratio of 10:1 (bacteria: cells), and the cells were incubated for 1 h at 25 °C. Finally, the cells were washed three times with M199, and 1 mL of 1% Triton X-100 was added to each well and mixed well. After gradient dilution, the colonies were counted, and the adsorption rate was calculated[42].

5.10 Challenge

To assess the pathogenicity of the three strains, LD_{50} values were determined for all strains[43]. First, the deletion strain Δ*Fis*, the complementation strain C-*Fis* and the wild-type strain TH0426 were inoculated in LB culture medium and cultured for 12 h. The bacterial colonies were counted, the concentration was calculated, and the bacterial solution was diluted 10-fold with sterile PBS. A total of 180 zebrafish were randomly divided into 3 groups with 6 gradients in each group. The bacterial solution from the previous step was used to inject the zebrafish intraperitoneally according to the corresponding gradient. The control group was injected with sterile PBS[44, 45]. After observation for one week, the number of deaths from the challenge was counted, and the LD_{50} of each strain towards zebrafish was calculated by the Kou method.

5.11 Expression of virulence genes

To analyse the cause of the change in virulence of the Δ*Fis* deletion strain, in this experiment, we screened 4 virulence genes for real-time quantitative PCR and detected the expression of these genes in Δ*Fis* and C-*Fis*[46].

5.12 Bacterial load test

Thirty healthy crucian carp were randomly divided into three groups, and the concentration of the bacterial solution was adjusted to 1×10^8 CFU/mL according to the above method. Crucian carp were intraperitoneally injected and inoculated, and the control group was injected with PBS. Three live fish were dissected in each group after 24 h and 72 h, and the blood, liver, kidney and spleen of each fish were ground. Then, the grinding droplet plate was placed on an RS solid plate, and incubated at 28 °C for 12 h, followed by colony counting. The whole process was conducted under sterile conditions.

5.13 Statistical analysis

Statistical analysis was performed with SPSS 16.0 software and GraphPad Prism version 8.0. For all tests, statistical significance was defined as $P < 0.05$. The results are expressed as the mean ± SD of at least three independent tests.

Abbreviations

CDC: Centers for Disease Control and Prevention; LB: Luria-Bertani; RS: Rimler-Shotts; pEASY: pEASY-blunt Zero; Amp: Ampicillin; Cm: Chloramphenicol; ORF: Open reading frame; OD600, OD575: Optical density; PBS: Phosphate Buffered Saline; EPC: Epithelioma papulosum cyprini; PCR: Polymerase Chain Reaction; cDNA: Complementary Deoxyribonucleic acid; RT-PCR: Reverse Time-Polymerase Chain Reaction; RNA: Ribonucleic Acid; LD_{50}: Median lethal dose; Medium 199: M199.

Declarations

The authors declare no comping interests.

Ethics approval and consent to participate

The study protocol was in accordance with the ethics guidelines of Jilin Agricultural University (JLAU) and the study was approved by the review board at JLAU, Changchun, China.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request. All data generated or analysed during this study are included in this published article.

Competing of interests

The authors declare that they have no competing interests.

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Authors' contributions

Study design: LXT and WJL. Study conduct, data collection and analysis: LXT. Data interpretation: SWW and SXF. Drafting of the manuscript: LXT. Revision of the manuscript content: SHC, ZDX, YBT, ZL and KYH. Approval of the final version of manuscript: QAD and SXF. LXT take responsibility for the integrity of the data analysis and interpretation. All the authors have read and approved the final version of the manuscript.

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Figures

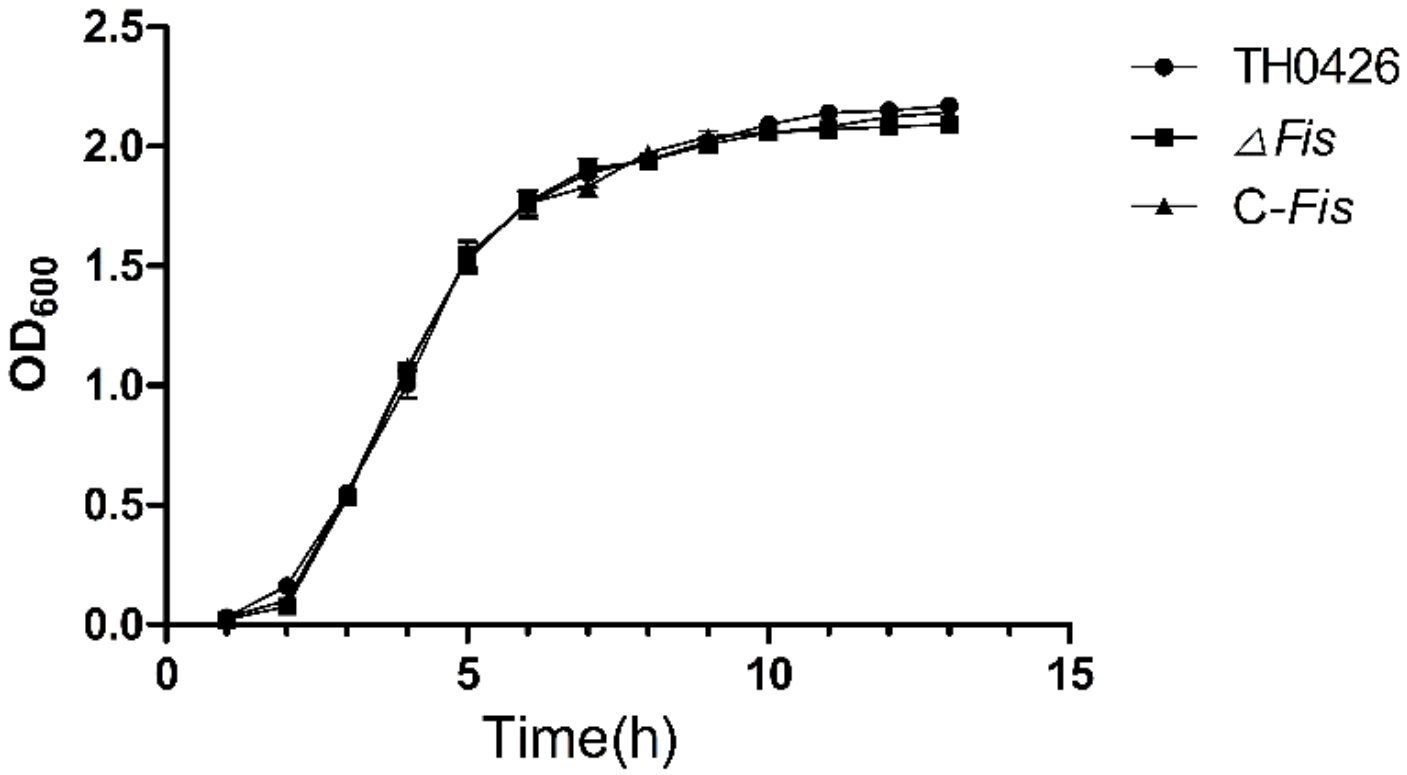

Figure 1

Growth curves of wild-type TH0426, ΔFis, and C-Fis.

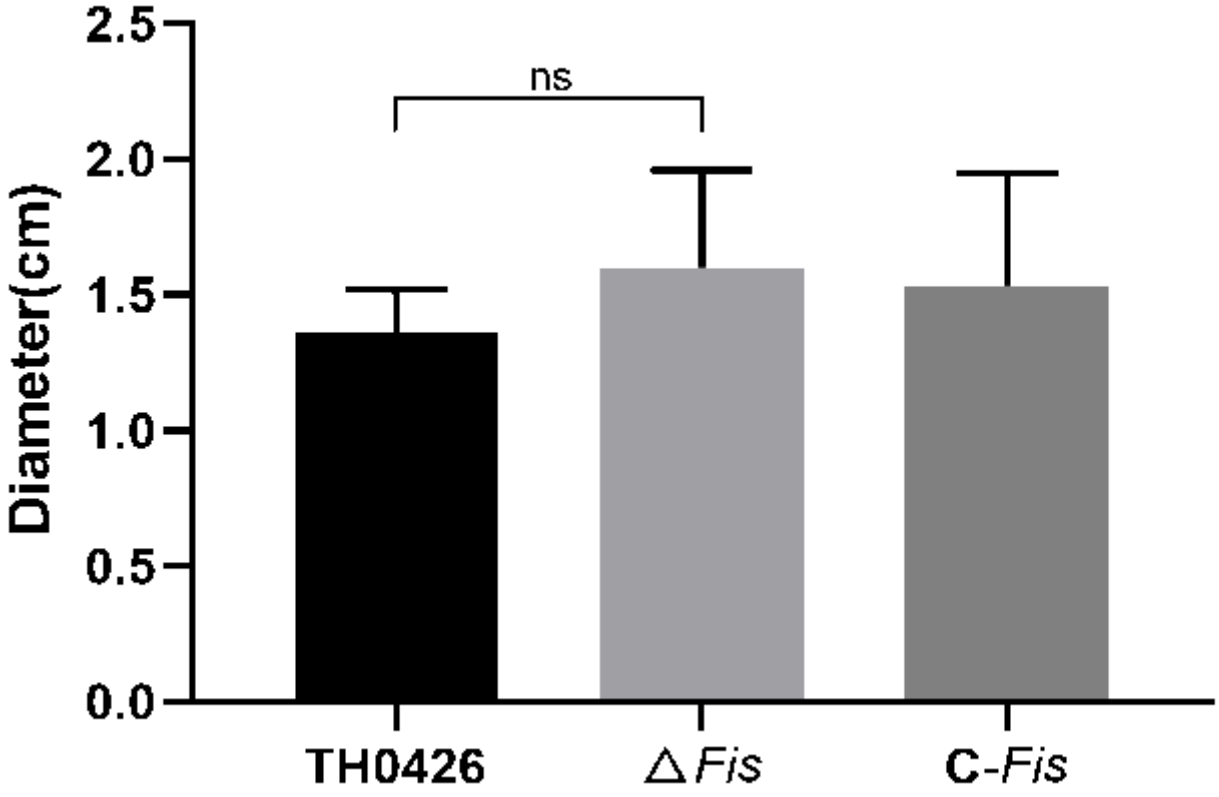

Figure 2

Swimming ability of wild-type TH0426, ΔFis, and C-Fis. (n.s. indicates no significant difference).

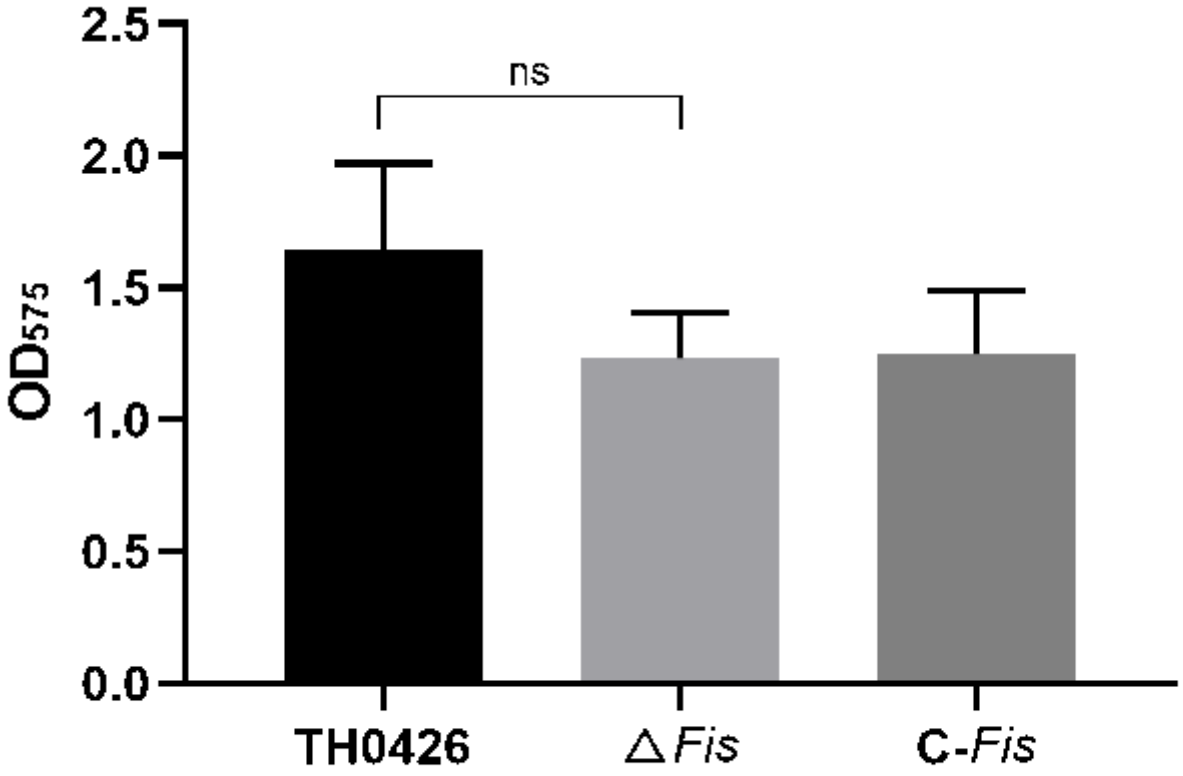

Figure 3

Biofilm formation ability of wild-type TH0426, ΔFis, and C-Fis. The amount of biofilm formation is represented by the OD575 value. (n.s. indicates no significant difference).

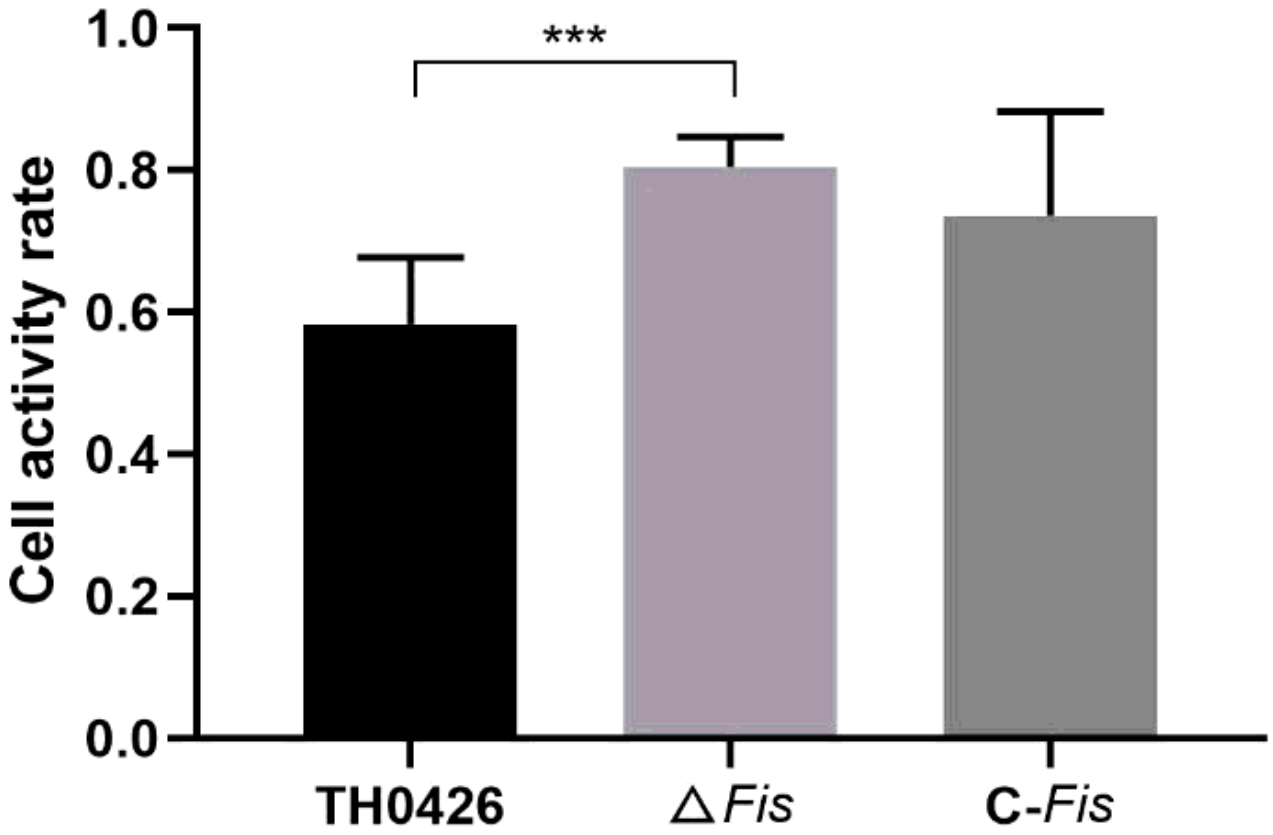

Figure 4

Cytotoxicity test of wild-type TH0426, ΔFis, and C-Fis. Since cell viability is directly proportional to the number of surviving cells, it is inversely proportional to cytotoxicity (*** indicates $P < 0.001$).

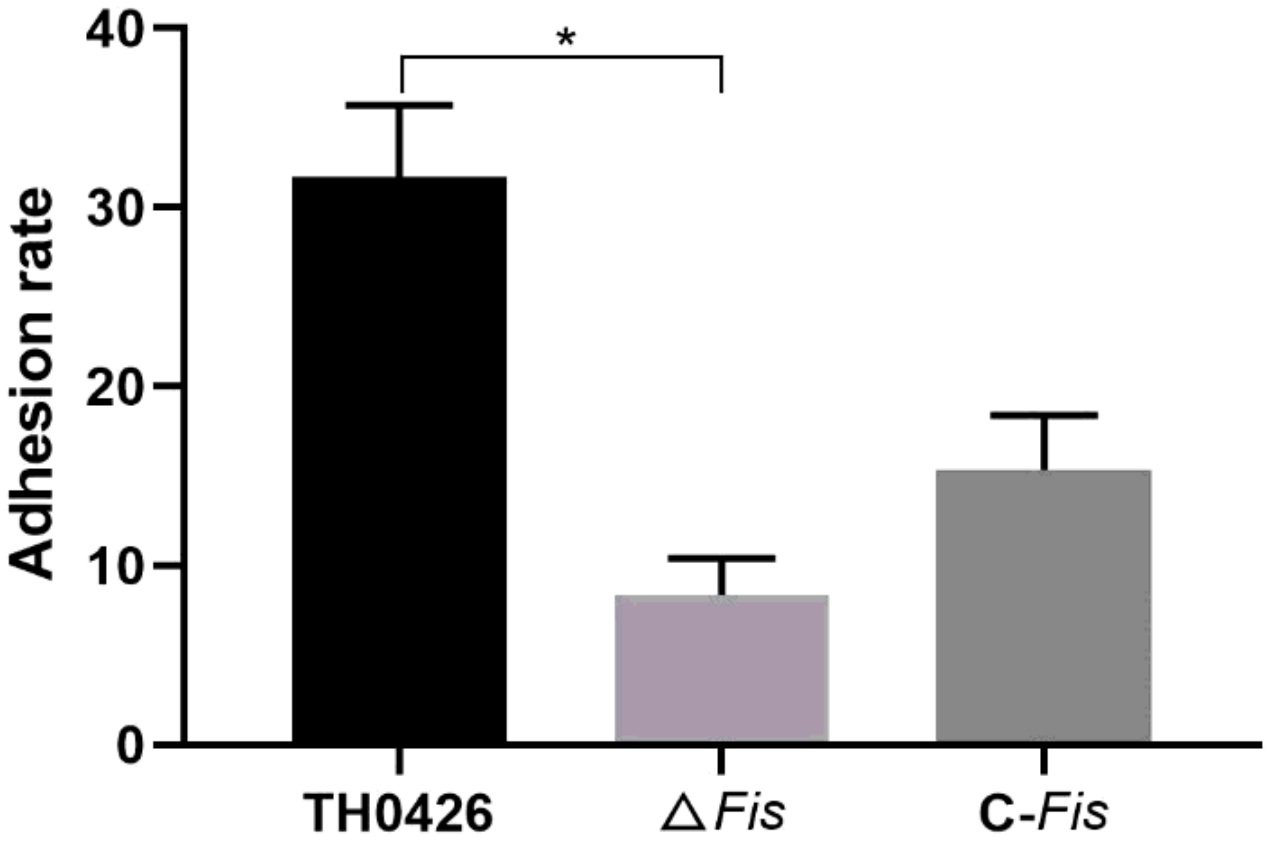

Figure 5

Detection of the adhesion and invasion abilities of wild-type TH0426, ΔFis, and C-Fis towards EPC cells. The data are expressed as the adherence rate (* indicates $P < 0.05$).

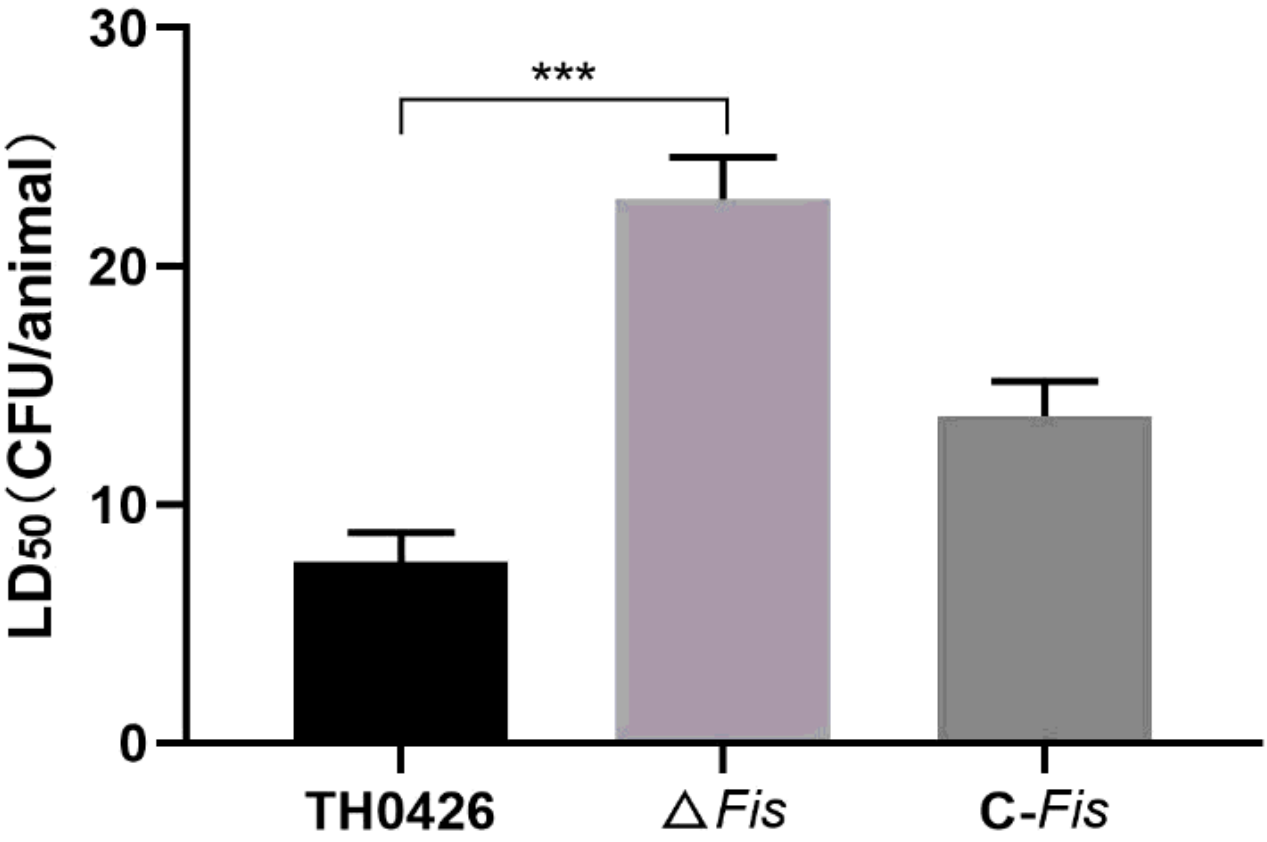

Figure 6

Survival rate of adult zebrafish challenged with wild-type TH0426, ΔFis, and C-Fis. The LD50 value represents the median lethal dose in zebrafish (*** indicates P < 0.001).

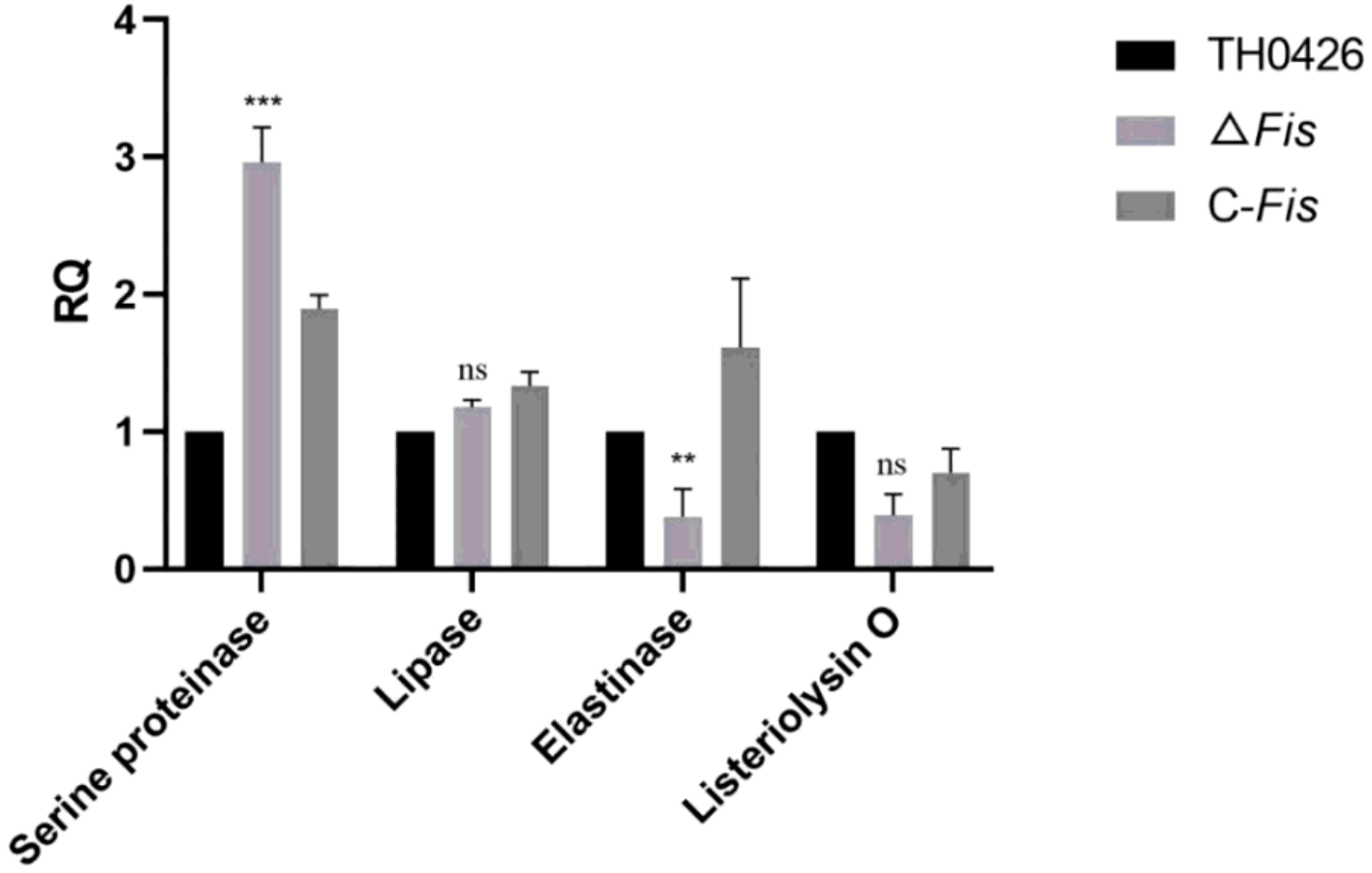

Figure 7

Expression of virulence genes in wild-type TH0426, ΔFis, and C-Fis.