

Original Research Articles

Deletion mutant, *ΔtssB* showed lower growth rate and lower antibacterial activity in *Flavobacterium columnare*

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Type VI secretion system (T6SS) in Gram-negative bacteria is involved in interbacterial competition. Although partial T6SS has been reported in *Flavobacterium columnare*, its possible function has never been characterized. In this study, one of the contractile sheath protein genes, *tssB* was cloned in *F. columnare* G₄ with 450 bp open reading frame (ORF) encoding 149 amino acids (aa) predicted to be 16.59 kDa. The *tssB* in *F. columnare* G₄ was phylogenetically clustered with the molecule in *F. columnare* ATCC 49512, and then with *F. johnsoniae*, implying that the T6SS in *F. columnare* may also belong to T6SSⁱⁱⁱ as reported in *F. johnsoniae*. With the available genetic manipulation technique, the knockout mutant, *ΔtssB* was constructed with the establishment of complementation strains, *tssB*⁺ strain and *tssB*⁺*tssC*⁺ strain. The *ΔtssB* showed similar colony morphology with rhizoids as observed in the wildtype *F. columnare* G₄, but exhibited significantly lower growth rate than the wildtype, and also lower antibacterial effect against *Aeromonas hydrophila* F4, as shown with the significantly lower concentration of *A. hydrophila* F4 in comparison with the wildtype. The differentially expressed genes (DEGs) of the *ΔtssB* strain comprised 13 upregulated and 102 downregulated genes, which were enriched in the Cellular Component term membrane and the nitrogen metabolism pathway, as revealed by GO and KEGG enrichment analyses. It is thus indicated that a potentially functional T6SS may exist in *F. columnare*, and further investigation should be conducted to reveal the exact composition of T6SS and its function in *F. columnare*.

1. INTRODUCTION

Flavobacterium columnare, a Gram-negative bacterium, is the causative agent of columnaris disease in a wide diversity of freshwater fish, including wild and farmed fish species.¹ Since its initial identification in the Mississippi River in the United States of America,² the causative pathogen has been continuously the focus of fish disease research in relation with genetic variety, epidemiology, immune response, possible treatment and vaccine development (e.g. Kumru et al.³; LaFrentz et al.⁴; Kayansamruaj et al.⁵; Abdelrahman et al.⁶; de Abreu Reis Ferreira et al.⁷; Frasin et al.⁸; Xiao et al.⁹; Jin et al.¹⁰; Sivarajan et al.¹¹). With the development of the gene deletion strategy,¹² type IX secretion system (T9SS), a unique secretion system present in the phylum Bacteroidetes,^{13,14} has been revealed as the virulence required for the pathogenicity of *F. columnare*.^{15,16} Recently, peptidase genes, including two

peptidase-encoding genes (C6N29_11545 and C6N29_11550) and *tspA*, which encodes a predicted tail-specific protease, have been confirmed as virulence factors of *F. columnare* using a deletion strategy or by disrupting T9SS.¹⁷

In Gram-negative bacteria, another secretion system, T6SS, which functions in competition and colonization over resources, has been widely reported,^{18,19} and T6SS can be classified into four distinct subtypes, namely T6SSⁱ to T6SS^{iv}.^{20,21} In the Bacteroidetes, the presence of T6SSⁱⁱⁱ has been reported,^{3,22} with its first report in *F. johnsoniae*.²³ However, in T6SSⁱⁱⁱ, some components, such as TssA, TssJ, TssL, and TssM were not reported, but they are functional in T6SSⁱ, and some unique components of T6SSⁱⁱⁱ, TssN, TssO, and TssP were hypothesized to compensate functionally for the missing proteins.²³ Recently, the assembly of five components, TssNQOPR was experimentally validated through protein-protein interaction.²² In a recent bioinformatic analysis on species in the *Flavobacterium*, a complete

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composition of T6SS, or the lack of a few components, such as *tssG*, *tssN*, *tssQ* in T6SS have been identified in different species in the genus.³ However, as a member of the Bacteroidetes, *F. columnare* has been reported to possess partial T6SS-encoding genes, but any attempt to understand the possible function of its T6SS and its effector proteins has so far remained poorly characterized.^{3,22,24}

It is known that TssB, a core structural protein of the T6SS, plays a critical role in system assembly and function.²⁵ The present study was thus designed to characterize the possible function of *tssB* in *F. columnare*. The gene deletion mutant and complementation strains were then established. The colony morphology, growth rate, antibacterial competition assay, and transcriptome sequencing were performed to examine the characteristics of this $\Delta tssB$ and to test if *tssB* is functional as a component of T6SS. The reduction in growth rate and in antibacterial competition against *Aeromonas hydrophila* F4 was then observed for $\Delta tssB$, which may imply the functional presence of T6SS in *F. columnare*.

2. MATERIALS AND METHODS

2.1. BACTERIAL STRAINS, PLASMIDS

Flavobacterium columnare G₄ was isolated previously and has been maintained for research in laboratory,^{12,26} and was cultured in Shieh liquid or solid medium at 28 °C.²⁷ *Aeromonas hydrophila*, isolated previously in the institute, and *Escherichia coli* were cultured in Luria-Bertani (LB) medium at 28 °C and 37 °C, respectively. Antibiotics used were based selectively on the resistance of plasmids or strains, and were purchased (Sangon Biotech), with 1 µg/mL tobramycin and 10 µg/mL tetracycline for *F. columnare*, and 100 µg/mL ampicillin for *E. coli*.

All bacterial strains and plasmids used in this study are listed in [Table 1](#).

2.2. BIOINFORMATICS ANALYSIS

Homologous genes were identified through local BLAST searches against the *F. columnare* G₄ genome (unpublished data) using BioEdit software. Nucleotide and amino acid sequences of target genes were analyzed via online BLAST platform (NCBI BLAST: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>; UniProt BLAST: <https://www.uniprot.org/blast>). Protein domain architecture and functional annotations were performed using the Pfam database (<http://pfam.xfam.org/>). Signal peptide sequences and cleavage sites were predicted using SignalP 6.0 (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>), while transmembrane domains were identified through Deep TMHMM 1.0 (<https://services.healthtech.dtu.dk/services/DeepTMHMM-1.0/>). Multiple sequence alignments of nucleic acid and protein sequences were generated using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Neighbor-joining (NJ) phylogenetic tree was generated using amino acid sequences with a bootstrap value of 10,000 times in MEGA 11 software.

2.3. CONSTRUCTION OF $\Delta TSSB$ DELETION MUTANT AND $TSSB^+$ AND $TSSB^+TSSC^+$ COMPLEMENTARY STRAINS

Gene deletion mutant of *F. columnare* was constructed as previously reported.^{12,15} The knockout plasmid pMS75-*tssB* was constructed as described in [Table 1](#) and subsequently transformed into *E. coli* S17-1 λ pir competent cells to serve as the donor strain for conjugation. The donor strain and the recipient *F. columnare* G₄ were harvested separately through centrifugation at 5,000 × g for 10 min at 25 °C, followed by two washes with 1 mL Shieh liquid medium, before being resuspended in 100 µL Shieh medium. The two strains were mixed, thoroughly vortexed, and spotted onto a 0.45 µm nitrocellulose filter membrane (NC membrane) (Millipore) placed at the center of a Shieh agar plate. After static incubation at 28 °C for 24 h, the conjugative bacterial lawn was streaked onto Shieh selection plates supplemented with 1 µg/mL tobramycin and 10 µg/mL tetracycline. The plates were incubated at 28 °C until single colonies appeared, yielding the first homologous recombination products. A single colony of the obtained recombinants was inoculated into 100 µL Shieh liquid medium and cultured with shaking for 48 h. The culture was then plated onto Shieh plates containing 5% sucrose for counter-selection. Following incubation at 28 °C until single colonies emerged, strains that underwent the second homologous recombination were obtained. Double-plate screening (Shieh plate vs Shieh plate supplemented with 5 µg/mL tetracycline, incubated at 28 °C for 12 h) was performed to identify tetracycline-sensitive clones. Finally, PCR was conducted using the out-flanking verification primers *tssBTF/tssBTR* to confirm the mutant strain. The successfully generated mutant was designated as $\Delta tssB$.

Complementation strains of *F. columnare* were also constructed using a strategy as previously described.^{12,15} The complementation plasmids pCP23-*tssB* and pCP23-*tssBtssC* were constructed as described in [Table 1](#). These plasmids were then separately transformed into *E. coli* S17-1 λ pir competent cells, which served as the donor strains for conjugation. The $\Delta tssB$ deletion mutant was used as the recipient strain. For conjugation, 1 mL each of donor and recipient cultures (OD₆₀₀ = 0.5) was collected and centrifuged at 5,000 × g for 10 min at room temperature. The cell pellets were washed twice by resuspension in 1 mL Shieh medium, followed by centrifugation. Finally, the donor and recipient cells were resuspended in 100 µL Shieh medium, thoroughly mixed, and spotted onto an NC membrane placed at the center of a Shieh agar plate. After incubation at 28 °C for 24 h, the bacterial lawn was spread onto Shieh selection plates supplemented with 1 µg/mL tobramycin and 10 µg/mL tetracycline. The plates were incubated at 28 °C until single colonies appeared. Single colonies were picked and inoculated into Shieh liquid medium containing 5 µg/mL tetracycline for further cultivation. The resulting complementation strains were designated as *tssB*⁺ strain and *tssB*⁺*tssC*⁺ strain, respectively.

All the primers used in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and are shown in [Table 2](#).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
<i>E. coli</i>		
DH5α	General cloning strain	Sangon Biotech
S17-1 λ pir	Conjugation strain	Lorenzo & Timmis(1994)
<i>F. columnare</i>		
G ₄	Wild type	Lu et al. ²⁶
<i>ΔtssB</i>	<i>tssB</i> deletion mutant of G ₄	This study
<i>ΔtssB/pCP23-tssB</i>	Complementation of <i>tssB</i> in <i>ΔtssB</i>	This study
<i>ΔtssB/pCP23-tssBtssC</i>	Complementation of <i>tssB</i> and <i>tssC</i> in <i>ΔtssB</i>	This study
<i>A. hydrophila</i>		
F4	Wild type; competitor	Li et al.(2011)
Plasmids		
pCP23	<i>E. coli</i> - <i>F. columnare</i> shuttle plasmid; Ap ^r (Tc ^r)	Agarwal et al.(1997)
pCP23- <i>tssB</i>	Construct used to complement <i>tssB</i> in <i>ΔtssB</i> ; <i>tssB</i> was amplified with primers <i>tssB</i> -For and <i>tssB</i> -Rev and cloned into BamHI and SphI sites of pCP23; Ap ^r (Tc ^r)	This study
pCP23- <i>tssBtssC</i>	Construct used to complement <i>tssB</i> and <i>tssC</i> in <i>ΔtssB</i> ; <i>tssB</i> and <i>tssC</i> were amplified with primers <i>tssB</i> -For and <i>tssB</i> -Rev2 and cloned into BamHI and SphI sites of pCP23; Ap ^r (Tc ^r)	This study
pMS75	<i>sacB</i> -containing suicide vector; Ap ^r (Tc ^r)	Li et al. ¹²
pMS75- <i>tssB</i> -up	2.1 kbp region upstream of <i>tssB</i> amplified with primers <i>tssB</i> -u-For and <i>tssB</i> -u-Rev and cloned into BamHI and Sall sites of pMS75; Ap ^r (Tc ^r)	This study
pMS75- <i>tssB</i>	Construct used to delete <i>tssB</i> in G ₄ ; 2.1 kbp region downstream of <i>tssB</i> amplified with primer <i>tssB</i> -down-For and <i>tssB</i> -down-Rev and cloned into Sall and PstI sites of pMS75- <i>tssB</i> -up; Ap ^r (Tc ^r)	This study

2.4. WESTERN BLOTTING ANALYSIS

Protein expression profile of various *F. columnare* strains were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB), following the previous protocol.¹² Specifically, the wildtype strain G₄, *ΔtssB* deletion mutant, *tssB*⁺ complementary strain, *tssB*⁺*tssC*⁺ complementary strain were separately inoculated into Shieh liquid medium (supplemented with 5 μg/mL tetracycline for complementary strains) and cultured under shaking until the logarithmic growth phase (OD₆₀₀ = 0.5). One milliliter aliquot of each culture was collected, and bacterial cells were separated from supernatants by centrifugation at 5000 × *g* for 10 min at 4 °C. The cell pellets were resuspended in 100 μL 1 × SDS loading buffer, while the supernatants were filtered through 0.22 μm membrane (Millipore) to remove residual cells. Subsequently, whole-cell proteins and extracellular proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore), which was blocked at room temperature for 1 h in tris-buffered saline (TBS) containing 5% skimmed milk. The membrane was then incubated with anti-TssB polyclonal antibody diluted 1:500 in TBS with 1% skimmed milk for 1 h at room temperature. The anti-TssB polyclonal antibody was generated in rats with the entire protein, MLSNHGIG GNEVPLDANEASEIPQNRITIAQKLTAESEPVKPELVEGLT

TIEKVFDHFKPEIKVDFENLDGSTKMENLNFKNLGDFGVKG ITQQSNFLTGLETEKDQYQKIIKQLKSNKILKGALEDPD AKKALLDSLQSLIKELEENK by a company (Wuhan Dai'an Biotechnology Co.). Then, the membrane was washed four times, each for 5 min, with TBST (TBS containing 0.1% Tween 20). Goat anti-rat IgG-HRP diluted at 1:5000 was added for 30 min incubation at room temperature, followed by four washes with TBST. Finally, chemiluminescent detection was performed, and images were obtained using a Bio-Rad ChemiDoc™ MP Imaging System.

2.5. GROWTH CURVE DETERMINATION AND COLONY MORPHOLOGY

The wildtype strain G₄ of *F. columnare* and its *ΔtssB* deletion mutant were streaked onto Shieh solid plates and incubated statically at 28 °C for 24 h. Single colonies were then inoculated into Shieh liquid medium and cultured with shaking at 28 °C and 180 rpm until the OD₆₀₀ reached 0.4. The cultures were sub-cultured at a 1:40 ratio into fresh medium (400 mL in an Erlenmeyer flask), thoroughly mixed, and 2 mL suspension was immediately collected to measure the initial OD₆₀₀ (recorded as 0 h). The flasks were incubated in a shaker at 28 °C (180 rpm), and the OD₆₀₀ was measured every hour until the experiment was terminated during a ten hour culture. Each experiment was performed in triplicate, and the mean values were used for analysis.

Table 2. Primers used in this study

Primer	Sequence (5'→3')
tssB-u-For	GCTAGGGATCCTCAATAAAATGGGTTTTTACA
tssB-u-Rev	GCTAGGTCGACCATAATACTTAATTTATTAGTTTAAC
tssB-down-For	GCTAGGTCGACTAAACAACTACTAATGGCACAAAAT
tssB-down-Rev	GCTAGCTGCAGTCATTTTCAGGAATAAGACTTAATAAAT
tssBTF	CTAACATTGGATCAGATTTTAT
tssBTR	TAATGGTAACATTTTTTACCTTGTC
tssB-For	CGCGGATCCACCTACCAATGCAAACTACTTGAAAT
tssB-Rev	CATGCATGCAATTTTGTGCTCCTTCAATAGTTG
tssB-Rev2	CATGCATGCTATGAGCTAAATAGATGTTAAGAAGTG
Fc16S-For	CAGTGGTGAAATCTGGT
Fc16S-Rev	GCTCCTACTTGCGTAGT
qF0	ACCCGATTACCATCGAAA
qR0	AGCTTGTAATTGGCAACGGT
qF1	GATGAGCGGTACCCAAACGA
qR1	TTAGGTTTTTGTGGGCGAC
qF2	ATGCCCCGATGGAGTACAAC
qR2	ACTACGGTCCTGTGGGTACA
qF3	ATGATGTGGGAATGGGGAGC
qR3	ACACTTGGGGTTGCATTGTT
qF4	ATACGGCGTACAGTTAGGCG
qR4	TGGGTACGATAGGTTGGTGC
qF5	CGACTTTTGGAGAGTGGTGGA
qR5	AGCCCTTTTACAAAAGCCTGT
qF6	GCAGATGGAAAAGTGGTGGC
qR6	GGTTTCGCCCTCTGGAATGT
qF7	CAGGAGGCGAAGTGATCGT
qR7	CTAAGGCAGCTGAAGGAGCA

Aliquots of early-log-phase cultures of the wild-type strain G_4 , $\Delta tssB$ mutant, were evenly spread onto Shieh agar plates and incubated statically at 28 °C until single colonies formed. Colony morphology was photographed using a Leica microscope (Germany).

2.6. ANTIBACTERIAL EFFECT OF $\Delta TSSB$ MUTANT

The wildtype strain G_4 of *F. columnare*, $\Delta tssB$ deletion mutant, $tssB^+$ single-complemented strain, $tssB^+tssC^+$ double-complemented strain, and the competitor wildtype strain *A. hydrophila* F4 were cultured to an OD_{600} of 0.4. Subsequently, 2 μ L of each *F. columnare* strains, and *A. hydrophila* F4, or a 1:1 (v/v) mixture of *F. columnare* and *A. hydrophila* F4 was spotted onto Shieh agar plates. The plates were incubated at 28 °C for 8 h, and afterward phenotypic observations were performed.

Following incubation, bacterial lawns were scraped from the plates, serially diluted, and plated for colony counting. The antibacterial effect of different *F. columnare* strains against *A. hydrophila* F4 was evaluated by comparing the colony-forming units (CFU) of the control group (*A. hy-*

drophila F4 alone) and the experimental group (*A. hydrophila* F4 co-cultured with *F. columnare* strains).

2.7. TRANSCRIPTOME ANALYSIS OF *F. COLUMNARE* WILDTYPE STRAIN AND $\Delta TSSB$ DELETION MUTANT

The wildtype strain G_4 of *F. columnare* and its isogenic $\Delta tssB$ mutant were streak-inoculated onto Shieh agar plates and cultured at 28 °C for 24 h. Single colonies were incubated at 28 °C with 180 rpm until reaching OD_{600} of 0.4. Bacterial cells were harvested and total RNA was extracted. RNA integrity and quantity were rigorously examined as being 9.50, 9.80, and 9.80 for three samples of the wild-type strain, and being 9.80, 9.80, and 9.80 for three samples of the $\Delta tssB$ mutant using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Following quality verification, a strand-specific library was constructed according to Parkhomchuk et al.²⁸ and validated using the Agilent 2100 Bioanalyzer. Qualified libraries were pooled based on effective concentration and target sequencing depth, followed by Illumina sequencing. The transcriptomic sequencing experiments were conducted in triplicates. Raw reads underwent quality control and filtering of low-quality se-

quences using fastp with default parameters. Clean reads were aligned to the *F. columnare* G₄ reference genome (unpublished data) using Bowtie2.²⁹ Transcriptome assembly and analysis were performed using Rockhopper.

Differential expressed genes (DEGs) analysis between the two groups were performed using the DESeq2 R package (version 1.20.0). Genes identified by DESeq2 with an adjusted *P*-value (padj) < 0.05 were designated as DEGs.³⁰ Gene Ontology (GO) enrichment analysis of DEGs was performed using the Goseq R package. Statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was analyzed using KOBAS software. DEGs were ultimately validated by quantitative real-time PCR (qRT-PCR).

2.8. QUANTITATIVE REAL-TIME PCR

For validation of differentially expressed genes, total RNA was extracted separately from *F. columnare* strains G₄ and $\Delta tssB$ mutant using the RNeasy® Mini Kit (Qiagen, Cat. No. 74104) according to the manufacturer's protocol. Extracted RNA was immediately reverse-transcribed into cDNA following DNase I treatment with gDNA Eraser (Takara, Japan), and cDNA synthesis was conducted using the PrimeScript™ RT Reagent Kit (Takara, Japan) in a 20 µL reaction system: 10 µL DNase-treated RNA, 1.0 µL PrimeScript RT Enzyme Mix I, 1.0 µL RT Primer Mix, and 4.0 µL 5× PrimeScript Buffer 2, with RNase-Free H₂O to final volume. Reactions started at 37 °C for 15 min, followed by 85 °C for 5 s.

The qRT-PCR was performed using iQ™ SYBR Green Supermix (Bio-Rad) on a CFX96™ Real-Time System (Bio-Rad) under following cycling conditions: 94 °C for 3 min; 45 cycles of 94 °C for 10 s, 54–60 °C for 20 s, 72 °C for 30 s, 80 °C for 5 s; followed by melting curve analysis (65–95 °C, 0.5 °C increments, 1 s/read). Reactions (20 µL) contained: 10 µL iQ™ SYBR Green Supermix (2×), 1.0 µL cDNA, 1.0 µL each forward/reverse primer (10 µM), and 7.0 µL H₂O. Gene expression changes were calculated using 2^{-ΔΔC_t} method, and normalized to 16S rDNA.

2.9. STATISTICAL ANALYSIS

To compare the difference in growth curves between *F. columnare* G₄ strain and $\Delta tssB$ mutant, two-sample *t*-test for area under the growth curve (AUC) comparison were employed as previously reported.³¹ Competition assays among two bacterial species were analyzed using a two-tailed Student's *t*-test, with *, **, ***, **** indicating *P* < 0.05, 0.01, 0.001, 0.0001.

3. RESULTS

3.1. SEQUENCE CHARACTERS OF *TSSB*

The *tssB* gene identified in *F. columnare* strain G₄ (GenBank accession no: PV745264) contains a 450 bp open reading frame (ORF) encoding 149 amino acids (aa), with a predicted molecular mass of 16.59 kDa. The TssB protein was predicted to have no signal peptide, no transmembrane

structure, and no conserved domain, but to be localized in bacterial cytoplasm (Fig. 1A). The amino acid sequence identity of TssB from *F. columnare* strain G₄ has 100% identity to FCOL_09740 (GenBank: AEW86757.1) of *F. columnare* ATCC 49512, which is predicted to be a small subunit of the contractile sheath of T6SS. The TssB has sequence identities of 75.2%, 48.7%, 20.4%, 20.1%, 16.3%, and 6.3% with its homologs in *Flavobacterium johnsoniae*, *Bacteroides fragilis*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Francisella tularensis*, and *Amoebophilus asiaticus*, respectively. Phylogenetic analysis indicated that TssB in *F. columnare* G₄ was clustered closely with the molecule in *F. columnare* ATCC 49512, and then with *F. johnsoniae*, a species known to possess T6SSⁱⁱⁱ (Fig. 1B).

3.2. CONSTRUCTION OF $\Delta TSSB$ MUTANT AND COMPLEMENTATION STRAINS

To investigate the function of *tssB*, the deletion mutant, $\Delta tssB$ was constructed in the wildtype *F. columnare* G₄, along with a *tssB* single-gene complementation strain, *tssB*⁺, and a *tssB*⁺*tssC*⁺ double-gene complementation strain, *tssB*⁺*tssC*⁺, as *TssB*⁺ and *TssC* are elements forming a complex together in T6SS.^{32,33}

The whole-cell proteins of these four strains were analyzed using Western blotting with a polyclonal antibody against the *TssB*⁺ protein (Fig. 2A). In the $\Delta tssB$ mutant, a band corresponding to the *TssB*⁺ protein was not detected, in contrast to the obvious presence of *TssB*⁺ in the wildtype *F. columnare* G₄. In *tssB*⁺ single-, and *tssB*⁺*tssC*⁺ double-complementation strains, a single band with similar molecular weight as in the wildtype strain was observed (Fig. 2A).

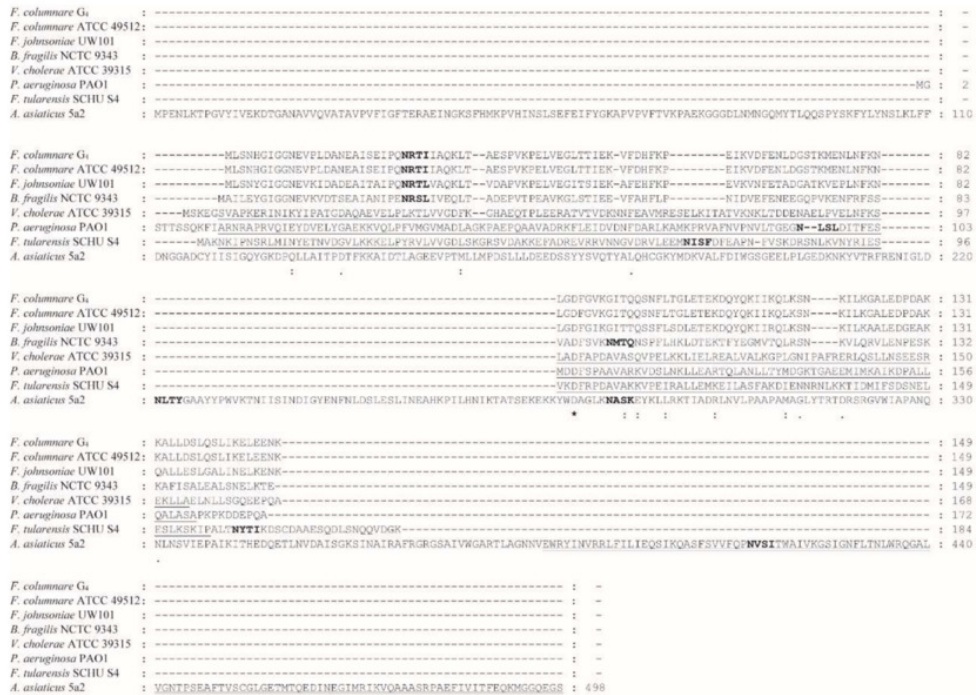
3.3. COLONY MORPHOLOGY, GROWTH AND TRANSCRIPTOME ANALYSIS OF $\Delta TSSB$ MUTANT

The wildtype G₄, $\Delta tssB$ mutant, and *tssB*⁺ all showed the typical well-developed rhizoid structure of colonies (Fig. 2B), similarly, as observed under a Leica microscope. The G₄ strain, $\Delta tssB$ mutant, *tssB*⁺ all exhibited similar colony morphology characteristic of a typical spreading growth pattern with dry central elevation, thin edges, and well-developed rhizoids (Fig. 2C). These results indicate that the deletion of *tssB* gene had no effect on morphological feature of *F. columnare* colonies.

Turbidimetric measurement was employed to monitor continuously the optical density variation of wildtype G₄ strain and $\Delta tssB$ mutant at different time points in order to examine the growth kinetic characteristics of the two strains (Fig. 2B). The wildtype G₄ strain had a significantly higher growth rate than $\Delta tssB$ mutant, indicating that the deletion of *tssB* gene substantially impairs bacterial growth (*P* < 0.001).

To understand the possible effect of *tssB* gene, transcriptome analysis was performed comparatively in $\Delta tssB$ mutant and the wildtype G₄ strain. The analysis of differentially expressed genes (DEGs; *P*-value (padj) < 0.05) revealed a total of 115 genes, with 13 upregulated and 102 downregulated genes (Supplementary Table 1; Fig. 3A).

1A



1B

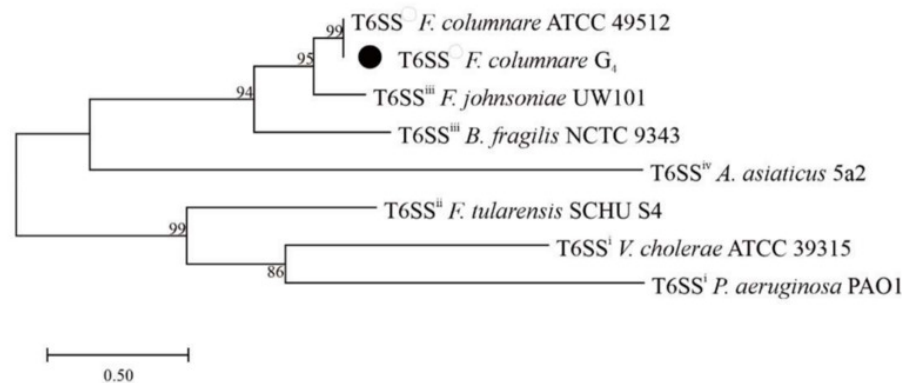


Figure 1. Multiple sequence alignment (A) and phylogenetic analysis (B) of predicted TssB amino acid sequences.

(A) Amino acid sequence similarity is indicated from high to low by *, : and ., respectively. Potential N-linked glycosylation sites are highlighted in bold. The conserved domain of T6SS VipA (PF05591) is underlined, whereas the phage sheath 1C domain (PF17482) is double-underlined. The strains used in the figure and their corresponding *tssB* locus tags are as follows: *Flavobacterium columnare* G₄ (PV745264), *F. johnsoniae* UW101 (FJOH_RS16940), *B. fragilis* NCTC 9343 (BF9343_1942), *V. cholerae* ATCC 39315 (VC_A0107), *P. aeruginosa* PAO1 (PA0083), *F. tularensis* SCHU S4 (FTT_1359c), and *A. asiaticus* 5a2 (Aasi_1074). (B) Phylogenetic tree of the contractile sheath protein TssB from *F. columnare* and other bacterial species. The tree was constructed based on predicted TssB amino acid sequences using NJ method implemented in MEGA 11, with 10,000 bootstrap replicates to assess branch support. Locus tags of the TssB sequences used in the phylogenetic analysis are the same in Figure 1A.

DEGs exhibiting a fold change > 2.83 included 13 upregulated genes, such as TonB-dependent receptor, MbnP family protein, and cytochrome-c peroxidase, and 102 downregulated genes included Sigma-54-dependent transcriptional regulator, zinc metalloprotease, MarR family winged helix-turn-helix transcriptional regulator, DUF4291 domain-containing protein, and *tssC* (Supplementary Table 1). DEGs were verified by qRT-PCR, among which PepSY domain-containing protein, TonB-dependent receptor, MbnP family protein, and cytochrome-c peroxidase were significantly upregulated, but *tssC* expression was downregulated (Fig. 3B).

GO functional enrichment analysis indicated that the DEGs were significantly enriched in the Cellular Component term, membrane, and heme binding (Fig. 4A). KEGG pathway enrichment analysis demonstrated significant enrichment of DEGs in the nitrogen metabolism pathway (Fig. 4B).

3.4. THE LOSS OF ANTIBACTERIAL EFFECT OF $\Delta tssB$ AGAINST *A. HYDROPHILA*

A wildtype *A. hydrophila* F4 strain was employed to examine the possible effect of $\Delta tssB$ mutant in combinations of com-

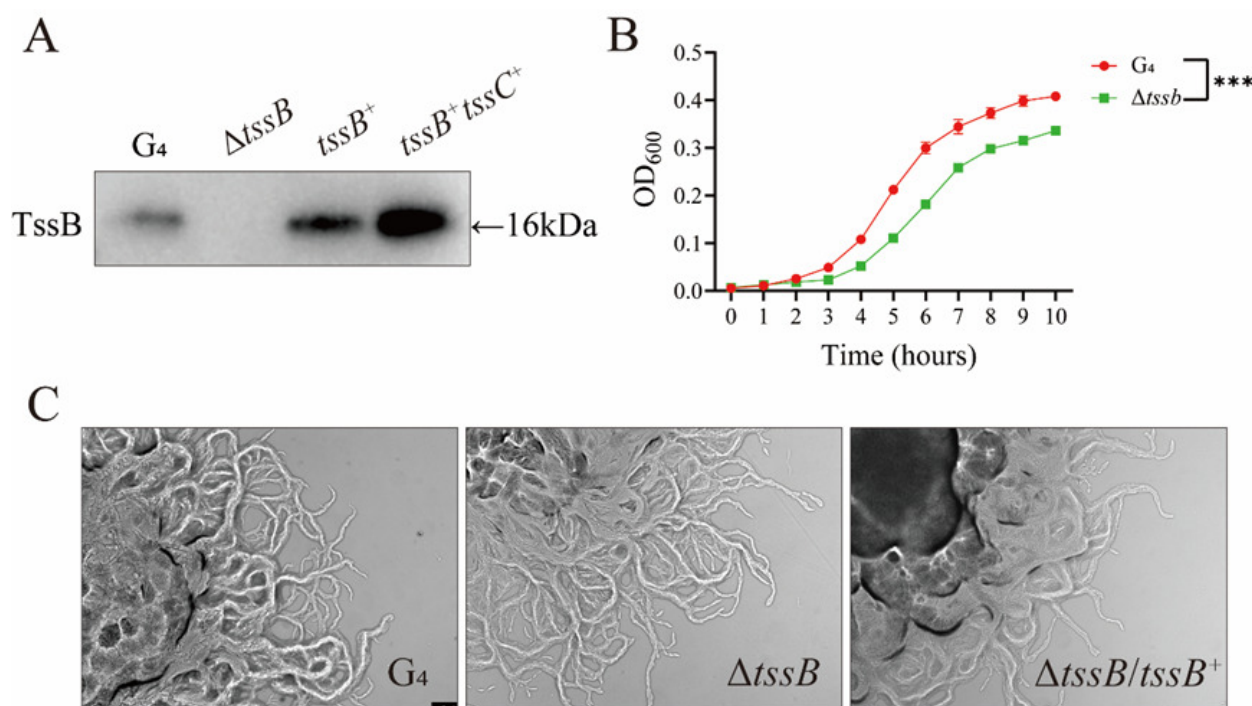


Figure 2. TssB protein detection (A), growth curve (B) and colony morphology (C) in wildtype, mutant and complementation strains of *F. columnare* G₄.

(A) Detection of TssB protein in whole-cell proteins of wildtype strain G₄, $\Delta tssB$, and complementation strains, *tssB*⁺ and *tssB*⁺*tssC*⁺. Whole-cell proteins were separated by SDS-PAGE and probed with anti-TssB polyclonal antibody. (B) Growth kinetics of *F. columnare* G₄ and $\Delta tssB$ in Shieh broth media, with OD₆₀₀ measured each hour until 10 h. Data were presented as mean $\pm$ SE, and analyzed with two sample *t*-test (*n* = 3), and *** indicating *P* < 0.001. (C) Colony morphology of *F. columnare* G₄ strains on Shieh agar. Scale bar = 75 μ m.

parative coculture (Fig. 5). It is obvious that when two wildtypes of *A. hydrophila* F4 and *F. columnare* G₄ were cocultured together, a significant reduction in bacterial concentration of *A. hydrophila* F4 was observed when compared with the single culture of *A. hydrophila* F4 alone (*P* < 0.001). The $\Delta tssB$ deletion mutant showed no such antibacterial effect, as no change in *A. hydrophila* F4 concentration was observed when cocultured (*P* > 0.05). The antibacterial effect of single complementation strain, *tssB*⁺ was not observed (*P* > 0.05); but the double complementation strain, *tssB*⁺*tssC*⁺, had significant antibacterial impact as shown by the lower concentration of *A. hydrophila* F4 (*P* < 0.001), although the concentration level was higher than the effect caused by wildtype G₄ (Fig. 5). These results thus indicated that the deletion of *tssB* resulted in the loss of antibacterial effect of *F. columnare*.

4. DISCUSSION

In this study, $\Delta tssB$, the deletion mutant of the *tssB* gene encoding a component of the contractile tail of T6SS^{3,23,25} was established in *F. columnare* G₄. The $\Delta tssB$ strain showed similar colony morphology as its wildtype with rhizoids, but had a significantly lower growth rate than the wildtype. It is obvious that the antibacterial effect of $\Delta tssB$ on growth, defined as the concentration of *A. hydrophila* F4, was reduced significantly when compared with wildtype *F. columnare* G₄, indicating the function of T6SS in *F. columnare* G₄.

It has been suggested that the rhizoid colony and the non-rhizoid colony of *F. columnare* may be related to their

virulence in infecting fish.^{7,34–36} Kunttu et al.³⁴ reported that *F. columnare* strains with rhizoids always had high virulence. Li et al.³⁵ reported the gene expression profiles of two strains, G₄ and G₁₈, of *F. columnare* with rhizoid and non-rhizoid colonies, respectively. In addition, different genomovars of *F. columnare* may be related to virulence,³⁶ and a recent identification of virulent *F. columnare* was also reported to have rhizoid colony morphology.⁷ *F. columnare* strains have been reported with large genetic diversity, and T9SS has been identified as the virulence factor for *F. columnare*, which is also related to bacterial gliding mobility for colonizing host tissues and for colony morphology.^{37,38} On the other hand, the other two species, *F. johnsoniae* and *F. psychrophilum* in the same genus, are also reported with rhizoid colony shapes for their virulent strains.^{34,39,40} However, the similar rhizoid colony morphology between $\Delta tssB$ and the wildtype may indicate that *tssB* is not related to the formation of rhizoid colony in *F. columnare*.

In a comparative genomic analysis, virulent strains of *F. columnare* were found to contain several spike proteins of T6SS, the valine-glycine repeat G (VgrG).²⁴ T6SS, which is widely present in Gram-negative bacteria, is typically recognized as an antibacterial weapon involved in interbacterial competition.²¹ As noted earlier, partial components of the T6SS have been identified in *F. columnare* in two previous reports.^{3,24} In *F. johnsoniae*, the T6SS was initially classified as T6SSⁱⁱⁱ because it differs from previously reported T6SSⁱ and T6SSⁱⁱ in the absence of TssA, TssL, TssM, and TssJ.²³ The T6SS in *F. columnare* has been identified

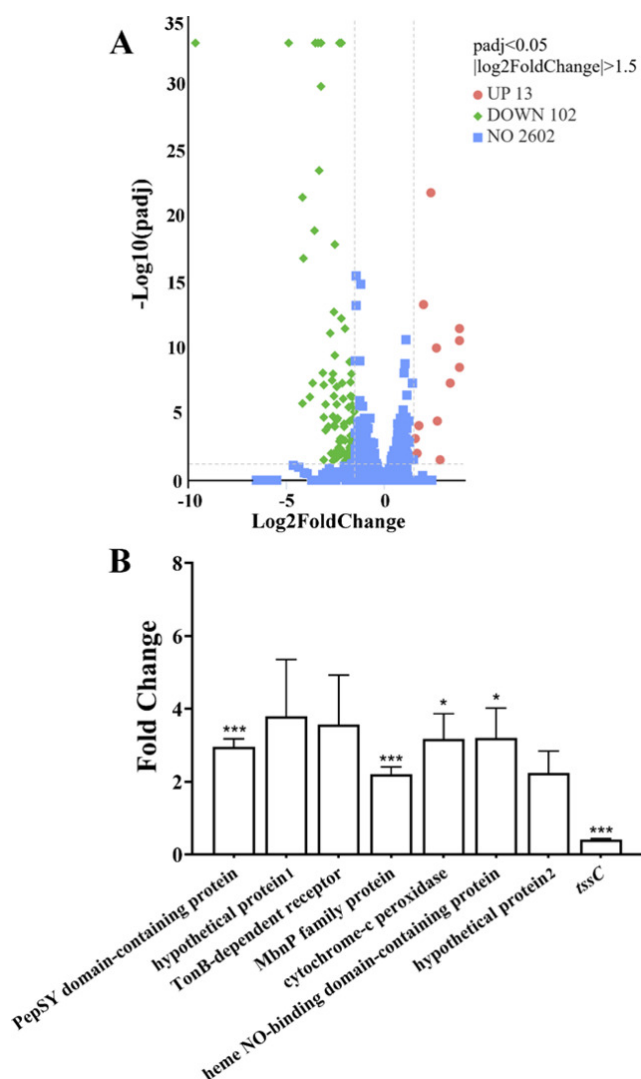


Figure 3. Differentially expressed genes (DEGs) between *F. columnare* G₄ and the *ΔtssB*.

(A) Red, green, and blue dots indicated those significantly upregulated genes, significantly downregulated genes, and non-significantly expressed genes, respectively. (B) Expression of eight DEGs in *F. columnare* G₄ and *ΔtssB*. Genes were detected by qRT-PCR. The sequencing experiments were performed in triplicates, and data were normalized by 16S rDNA and expressed as mean $\pm$ SE, with * and *** indicating $P < 0.05$ and $P < 0.001$.

as T6SS^{iii,3,24} as supported by the phylogenetic relationship between TssB in *F. columnare* and *F. johnsoniae* in the present study. With the availability of gene manipulation technique,¹² the mutant, *ΔtssB*, was found to show significantly reduced growth rate, and also a defect in bacterial competitiveness, indicating at least a certain degree of functional loss of T6SS in the mutant. However, it should be emphasized that the exact gene composition of T6SS in *F. columnare* needs to be decoded and its role in bacterial competition requires further investigation. Whether the deficiency of *tssB* in *F. columnare* is related to the bacterial virulence also requires further *in vivo* investigation.

To further investigate the mechanism underlying the differential bacterial competitiveness between the wildtype *F. columnare* G₄ and its *ΔtssB* deletion mutant, transcriptome sequencing revealed 13 upregulated and 102 downregulated genes. Although the possible function of these genes has not been examined in *F. columnare*, some of these

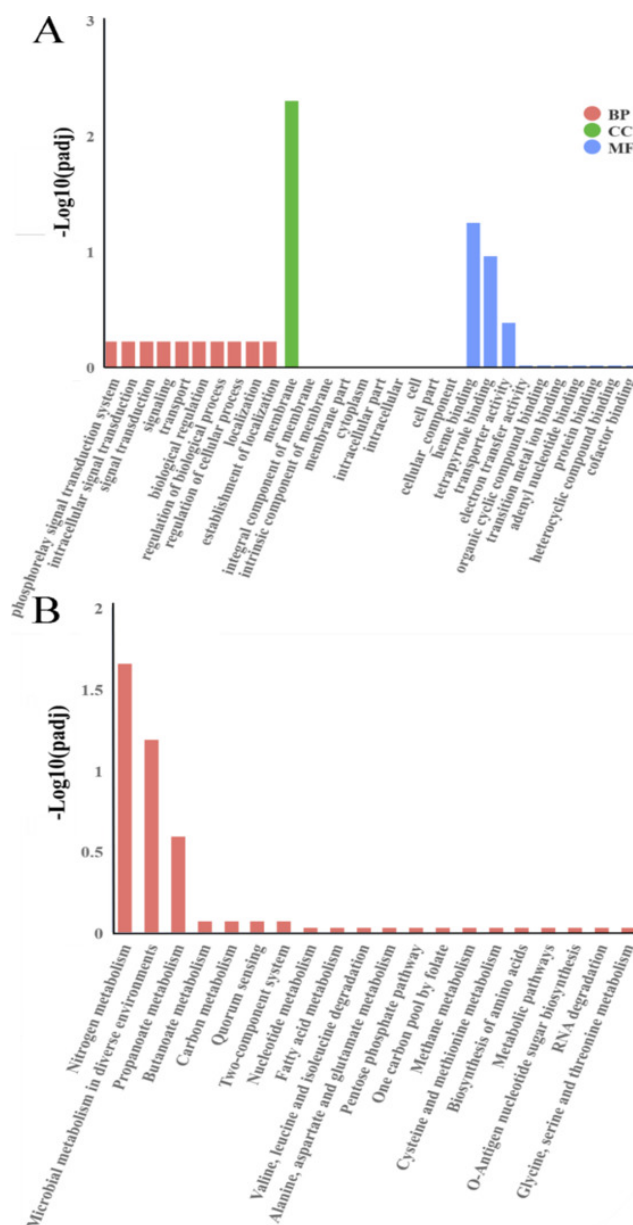


Figure 4. Enrichment (A) and KEGG pathway enrichment (B) of DEGs between wildtype *F. columnare* G₄ and *ΔtssB*.

BP denotes Biological Process, CC denotes Cellular Component, and MF denotes Molecular Function. The y-axis represented the significance level of enrichment and was expressed as $-\log_{10}(\text{padj})$.

genes, as reported in other bacteria, may indicate their possible relationship with T6SS. Durán et al.⁴¹ reported that in *Pseudomonas fluorescens* F113, FleQ, a member of the NtrC family can activate the expression of the F1-T6SS and F3-T6SS gene clusters by sensing c-di-GMP signals and nitrogen source status. This activation enhances interbacterial competitive ability (killing other bacterial strains) and rhizosphere colonization efficiency. In *Burkholderia pseudomallei*, the inactivation of *tctR*, a MarR family transcriptional regulator, resulted in an increase in *hcp2-lacZ* transcriptional fusion expression, indicating that TctR acts as a repressor of the T6SS-2 gene cluster.⁴² In *Yersinia pseudotuberculosis*, HpaR, another MarR family transcriptional regulator, can positively regulate the expression of T6SS4

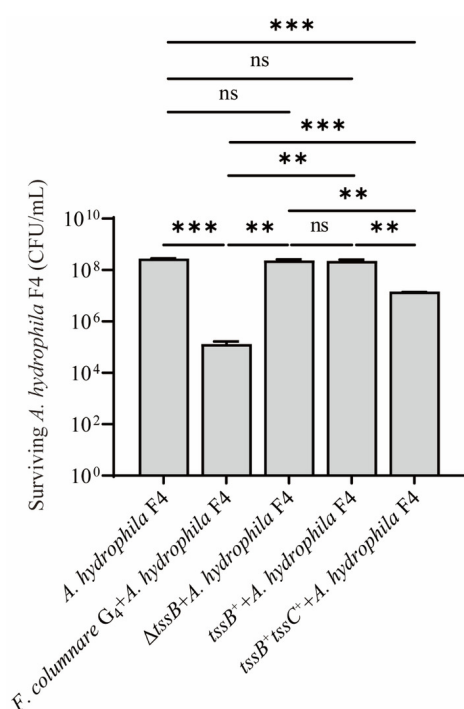


Figure 5. Bacterial competition assay between *F. columnare* G₄, $\Delta tssB$, *tssB*⁺, *tssB*⁺*tssC*⁺ and *Aeromonas hydrophila* F4.

Surviving *A. hydrophila* F4 cocultured with *F. columnare* strains were measured through serial dilutions and colony counting. Data were expressed as mean $\pm$ SE from three independent experiments. The two-tailed Student's *t*-test was used to determine the statistical significance, with ** indicating $P < 0.01$, and *** $P < 0.001$.

in response to oxidative stress.⁴³ MarR family regulators are typically involved in the regulation of virulence factor synthesis and aromatic compound catabolism,⁴⁴ and the observed downregulation of MarR regulators may reflect their role in normal metabolism, thereby accounting for the lower growth rate of $\Delta tssB$ in the present study. Whether the downregulated sigma-54-dependent transcriptional regulator, which belongs to the NtrC family, and the MarR family winged-helix-turn-helix transcriptional regulators function as transcriptional regulators for *F. columnare* requires further investigation. In addition, the DUF4291 domain was reported to show high homology with ART-like effector proteins in pathogenic *Legionella* species,⁴⁵ but whether the DUF4291 domain in *F. columnare* is an effector of T6SS also requires further investigation.

A few other downregulated genes may provide a hypothetical basis for explaining the lower growth rate of the $\Delta tssB$ mutant in the present study. The deletion of *tssB* and downregulation of *tssC* in the mutant might have resulted in the disruption of the T6SS sheath, as TssB and TssC are two components of the T6SS sheath.²¹ It may be possible that the loss of the T6SS sheath and probably the T6SS organelle may have an influence on the bacterial membrane, and on the growth rate of the $\Delta tssB$ mutant. On the other hand, the research on genes enriched in heme-binding function is rather limited in *F. columnare*, but available literature may indicate that T6SS is probably related to heme-binding and heme transportation. In *Burkholderia thailandensis*, the outer membrane heme transporter,

HmuR was reported to interact with TseZ, a proteinaceous zincophore secreted through T6SS for zinc acquisition.⁴⁶ In *Pseudomonas aeruginosa*, the oxygen-binding hemerythrin, Mhr, is secreted as an effector of the H2-T6SS and interacts with cbb3-type cytochrome c oxidase subunits, thereby influencing intracellular NADH/NAD⁺ levels.⁴⁷ Collectively, these processes may influence bacterial growth. In the present study, the function of two upregulated genes, *cytochrome-c peroxidase* and *heme NO-binding domain-containing protein*, and some downregulated genes, such as *hmpA*, and *cytochrome c*, which were enriched in heme-binding, is unknown in *F. columnare* and requires further investigation.

In the present study, the downregulated genes enriched in nitrogen metabolism as revealed in KEGG include *cytochrome c*, *cbb3-type cytochrome c oxidase subunit I*, *nirK*, *nitrate reductase*, *MFS transporter*, some of which may have a relation with T6SS or with nitrogen metabolism as reported in other bacteria. As stated above, *cbb3-type cytochrome c oxidase* may have a relation with T6SS in *Pseudomonas aeruginosa*.⁴⁷ In *B. pseudomallei*, the downregulation of T6SS is related to the reduction of nitrate reductase operons.⁴⁸ The *nirK* is involved in bacterial denitrification.⁴⁹ However, the exact mechanism of these genes enriched in nitrogen metabolism should be of interest for future research in *F. columnare*.

In summary, the mutant, $\Delta tssB$, with the deletion of the *tssB* gene encoding a contractile tail component of T6SS showed reduced growth rate and reduced competitiveness when compared with the wildtype, indicating the functional presence of T6SS in *F. columnare*. The exact composition of the T6SS in *F. columnare*, its transcriptional regulation, and its effector proteins should be of interest for further investigation.

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alization: Nan Li (Lead). Writing – review & editing: Bo Li (Equal), Pin Nie (Equal).

ETHICAL CONDUCT APPROVAL – IACUC

All animal experimental procedures were approved with permission number 20240910 from the School of Marine Science and Engineering, Qingdao Agricultural University, and conducted in strict accordance with its guidelines for the care and use of laboratory animals.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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SUPPLEMENTARY MATERIALS

3176681 Supplementary Table

Supplementary Table of Up-regulated and Down-regulated Genes

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