The Emerging Fish Pathogen 
*Flavobacterium spartansii* Isolated from Chinook Salmon: Comparative Genome Analysis and Molecular Manipulation

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*Flavobacterium spartansii* strain T16 was isolated from a disease outbreak in hatchery-reared Chinook salmon (*Oncorhynchus tshawytscha*) fingerlings. To gain insight into its genomic content, structure and virulence pathogenesis factors, comparative genome analyses were performed using genomes from environmental and virulent *Flavobacterium* strains. *F. spartansii* shared low average nucleotide identity (ANI) to well-known fish-pathogenic flavobacteria (e.g., *F. columnare*, *F. psychrophilum*, and *F. branchiophilum*), indicating that it is a new and emerging fish pathogen. The genome in T16 had a length of 5,359,952 bp, a GC-content 35.7%, and 4,422 predicted protein-coding sequences. *Flavobacterium* core genome analysis showed that the number of shared genes decreased with the addition of input genomes and converged at 1182 genes. At least 8 genomic islands and 5 prophages were predicted in T16. At least 133 virulence factors associated with virulence in pathogenic bacteria were highly conserved in *F. spartansii* T16. Furthermore, genes linked to virulence in other bacterial species (e.g., those encoding for a type IX secretion system, collagenase and hemolysin) were found in the genome of *F. spartansii* T16 and were conserved in most of the analyzed pathogenic *Flavobacterium*. *F. spartansii* was resistant to ampicillin and penicillin, consistent with the presence of multiple genes encoding diverse lactamases and the penicillin-binding protein in the genome. To allow for future investigations into *F. spartansii* virulence in vivo, a transposon-based random mutagenesis strategy was attempted in *F. spartansii* T16 using pHimarEm1. Four putative gliding motility deficient mutants were obtained and the insertion sites of pHimarEm1 in the genome of these mutants were characterized. In total, study results clarify some of the mechanisms by which emerging flavobacterial fish pathogens may cause disease and also provide direly needed tools to investigate their pathogenesis.

**Keywords:** *Flavobacterium spartansii*, genome analysis, virulence factors, mutation
INTRODUCTION

Among the fish diseases that result in significant losses in farmed and wild fish populations around the world, those caused by flavobacteria (Family Flavobacteriaceae, Phylum Bacteroidetes) occupy a central position (Starliper and Schill, 2011). Although the majority of published reports on flavobacterial disease outbreaks in fish, along with the research aimed at combatting them, has focused on three Flavobacterium spp. (Flavobacterium psychrophilum, F. columnare, and F. branchiophilum) (Starliper and Schill, 2011; Loch and Faisal, 2017), multiple novel Flavobacterium spp. have recently emerged in association with fish disease outbreaks (reviewed in Loch and Faisal, 2015). Interestingly, some of these novel flavobacteria not only cause systemic disease outbreaks in fish, but also generate disease signs that mimic their better-known fish-pathogenic counterparts (Loch and Faisal, 2016), thereby complicating disease diagnosis and treatment. Unfortunately, very little is known about the disease ecology, transmission, and pathogenesis of emergent fish-associated flavobacteria.

Despite decades of research, effective means for the prevention and control of flavobacterial diseases have yet to be realized. However, with the advancement and ready availability of complete genome sequencing technologies for microbes, there have been significant efforts to elucidate the molecular mechanisms behind flavobacterial pathogenicity and thereby guide vaccine development through genome sequencing of pathogenic and environmental flavobacteria alike. Indeed, complete genomes are available for a handful of Flavobacterium spp. (McBride et al., 2009; Barbier et al., 2012; Kumru et al., 2017) and additional genome sequence data are now available for multiple strains of the fish pathogens F. columnare (Bartelme et al., 2016; Zhang et al., 2016), F. psychrophilum (Wiens et al., 2014; Wu et al., 2015), and F. branchiophilum (Touchon et al., 2011). These studies have revealed some of the processes by which flavobacterial fish pathogens are believed to colonize, attack, and survive within fish hosts (Duchaud et al., 2007; Castillo et al., 2016), as well as how some of the molecular mechanisms vary amongst strains (Wiens et al., 2014; Wu et al., 2015; Bartelme et al., 2016; Zhang et al., 2016).

Recently, we described the novel species Flavobacterium spartansii, which was originally isolated from a disease outbreak in hatchery-reared Chinook salmon (Oncorhynchus tshawytscha) fingerlings and from systemically-infected, feral, spawning Chinook salmon (Loch and Faisal, 2014a). Subsequent studies investigating the pathogenicity of two F. spartansii strains (T16 and S12) under laboratory conditions showed that they were capable of inducing morbidity and mortality in intraperitoneally injected Great Lakes Chinook salmon, although the estimated median lethal dose was relatively high and varied by strain (Loch and Faisal, 2016). Nevertheless, the induced gross and microscopic changes were severe at times, which may suggest that F. spartansii is a facultative salmonid pathogen. Therefore, a detailed examination of its possible pathogenicity is warranted. Through functional and comparative genomic analyses, the following study aimed to: (a) better understand the mechanisms of virulence for F. spartansii and investigate how its genetic repertoire compares with the better-known fish-pathogenic flavobacteria through the use of complete genome sequencing; (b) examine the antimicrobial resistance capabilities in F. spartansii; (c) further characterize the evolution of virulence among Flavobacterium species; and (d) develop additional molecular tools for genetic manipulation of flavobacteria.

MATERIALS AND METHODS

Culture Conditions

Flavobacterium spartansii (T16T) was grown aerobically in CYE broth. Escherichia coli DH5α was used for cloning and plasmid propagation. E. coli S17 (λ, pir) was used for conjugation. E. coli EC100D pir+ was used to recover transposons from F. spartansii. E. coli strains were routinely grown in Luria-Bertani (LB) broth. Liquid cultures were incubated with shaking (ca. 200 rpm) at either 28°C (F. spartansii) or 37°C (E. coli). For solid LB medium, Bacto agar (Difco, Detroit, MI) was added at a final concentration of 20 g/liter with kanamycin (50 µg/ml) or ampicillin (100 µg/ml) added for plasmid selection in E. coli or erythromycin (Em) added (100 µg/ml) for transposon or plasmid selection in F. spartansii. Demonstration of motility was done by culturing F. spartansii cells on PY2 agar medium at 28°C.

Molecular Manipulation

Isolation and purification of bacterial genomic DNA was performed with the Wizard Genomic DNA Purification Kit (Promega, CA, USA) according to the manufacturer’s instruction. The purity, quality and quantity of purified genomic DNA were assessed using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific, MA, USA) and a Qubit 2.0 fluorometer (Life Technologies, MA, USA), respectively.

Random Transposon Mutagenesis

Random transposon mutagenesis in F. spartansii T16T was performed using pHimarEm1 (Braun et al., 2005). Donor E. coli S17-1 λ, pir cells with pHimarEm1 and recipient cells T16T were grown to the log phase. Cells were collected by centrifugation at 3,800 rpm for 15 min. F. spartansii T16T and E. coli cells were mixed at 1:1 ratios in CYE medium supplemented with Ca2+ and spotted onto CYE agar. After 24-h incubation at 28°C, the mixed cells were scraped from agar plates, washed once with CYE broth, and plated on the CYE agar containing erythromycin (100 µg/ml). Next, erythromycin transconjugants were transferred to PY2 agar (8%) for screening the spreading-deficient mutants. The gliding deficiency mutants were further observed for their motility on glass slides under microscopy. The pHimarEm1 insertion sites in the non-spreading mutants were identified using a published method (Braun et al., 2005). Briefly, genomic DNA was digested by XbaI overnight, purified with Qiagen PCR purification kit, and self-ligated. The ligation mixture was electroporated into E. coli Ec100D+ ( Epicentre Technologies). Purified plasmid DNA from the Km-resistant colonies was sequenced using the primer Walker85 (TGGGAAATCATTGGAAGGTTGG) and Walker86 (TCGGGTATCGCTTCTGAGG). The sequences
were blasted against Flavobacterium spartansii T16T genome to characterize the insertion site. Growth between the selected mutants and WT was compared by determination of the OD600nm after overnight culturing in PY2 broth (Braun et al., 2005).

**Hemolysin Analysis**

Hemolysin production in Flavobacterium spartansii T16T was examined by inoculating bacteria on Remel Blood Agar (Thermo Scientific, KS). Hemolytic activity was evaluated following incubation at 28°C for 48 h. The controls for α- and β-hemolysin producers were Elizabethkingia meningoseptica ATCC 13253 and Staphylococcus aureus MSU001, respectively (Chen et al., 2017).

**Antibiotic Susceptibility Testing**

Flavobacterium spartansii isolates T16T and S12 were tested for antibiotic susceptibility using the Kirby-Bauer disk diffusion method as previously described (Locht and Faisal, 2014b). In brief, cultures grown on Hsu-Shotts medium (48 h) were re-suspended in sterile 0.85% saline and adjusted to OD600 nm of 0.5 in a Biowave CO8000 Cell Density Meter (WPA Inc., Cambridge, UK). The bacterial suspension in duplicate was inoculated onto dilute Mueller-Hinton agar (Hawke and Thune, 1992) without 5% calf serum. Antibiotic-impregnated disks were stamped onto the medium and plates incubated at 22°C for 48 h, at which time the zones of inhibition were measured and means calculated. Antibiotics included polymyxin-B (PB; 300 µi), oxytetracycline (T; 30 µg), trimethoprim-sulfamethoxazole (SXT; 25 µg), erythromycin (E; 15 µg), ampicillin (AMP; 10 µg), florfenicol (FFC; 30 µg), penicillin G (P; 10 µi), and the vibriostatic agent O/129 (O129, 2,4-diamino,6,7-di-isopropyl pteridine; 10 µg). The cutoffs for sensitivity were as follows: PB < 8 mm resistant; 8–12 mm intermediate sensitivity; >12 mm sensitive; T < 15 mm resistant; 15–18 mm intermediate sensitivity; >18 mm sensitive; SXT < 11 mm resistant; 11–15 mm intermediate sensitivity; >15 mm sensitive; E < 14 mm resistant; 14–18 mm intermediate sensitivity; >18 mm sensitive; AMP < 12 mm resistant; 12–13 intermediate sensitivity; >13 mm sensitive; FFC < 16 mm resistant; 16–21 mm, intermediate sensitivity; >21 mm, sensitive; O/129, < 7 mm resistant, >7 mm sensitive (Whitman, 2004).

**Genome Sequencing, Assembly, and Annotation**

Next generation sequencing (NGS) libraries were prepared using the Illumina TruSeq Nano DNA Library Preparation Kit. Completed libraries were evaluated using a combination of Qubit dsDNA HS, Caliper LabChipGX HS DNA, and Kapa Illumina Library Quantification qPCR assays. Libraries were combined in a single pool for multiplexed sequencing and this pool was loaded on one standard MiSeq flow cell (v2) and sequencing was performed in a 2 × 250 bp paired end format using a v2, 500 cycle reagent cartridge. Base calling was done by Illumina Real Time Analysis v1.18.54 and the output of RTA was demultiplexed and converted to a FastQ format with Illumina Bcl2fastq v1.8.4 (Klein et al., 2013). NGS libraries were sequenced by Illumina Miseq paired-end sequencing technology at the Research Technology Support Facility (RTSF) at Michigan State University.

The reads were assembled using SPAdes (version 3.9.0. Gene annotation was carried out by NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP 3.3) (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Initial prediction and annotation of open reading frames (ORF) and tRNA/rRNA gene prediction were carried out with Glimmer 3 through the Rapid Annotation using Subsystem Technology server (RAST).

**Bioinformatics**

The functional categorization and classification for predicted ORFs were performed by RAST server-based SEED viewer (Aziz et al., 2008). The multi-drug resistance genes were predicted by Resistance Gene Identifier (RGI) tool implemented in CARD website (McArthur et al., 2013). Determination of phage elements was conducted in PHASTER (Arndt et al., 2016). Identification of genomic islands was performed in IslandViewer3 using F. johnsoniae genome as the reference (http://www.pathogenomics.sfu.ca/islandviewer). Prophage and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) were predicted using the CRISPRFinder (Grissa et al., 2007). Detection and identification of virulence factors were carried out using MvirDB database (Zhou et al., 2007). Some virulence factors found in Bacteroidetes were manually compared among those fish pathogens and environmental isolates (Chen et al., 2005). For genome similarity assessment, digital DNA-DNA hybridization (dDDH) values were computed using web tool GGDC 2.1 (formula 2, identities/HSP length). Further, the average nucleotide identity (ANI) values were computed by ANI calculator (http://enve-omics.ce.gatech.edu/ani/).

The pan genome, core genome, and specific genes of Flavobacterium spartansii were analyzed by comparison with 13 representative Flavobacterium using EDGAR 2.0 (Blom et al., 2016). The sizes of pan genome and core genomes were approximated using the core/pan development feature.

**Data Deposition**

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession MK00000000. The version described in this paper is version MK00000000. The BioProject designation for this project is PRJNA341797. BioSample accession number is SAMN05728473.

**RESULTS**

**Genome Features and Phylogenic Inference**

The general genome features of Flavobacterium spartansii compared to those representative Flavobacterium, and assembly statistics, are presented in Table 1. The assembly of Flavobacterium produced 5.35 Mb of sequence across 29 contigs with an N50 of 515,812 bp, the longest sequence of 1,308,521 bp and a G+C content of 36% (Table 1). The genome was predicted to have at least 4,422 protein-coding genes and 130 RNA genes. Among the protein-encoding genes, 2,864 of them could be assigned a putative function, whereas 1,772 genes were predicted to encode
hithetical proteins. At least 2,119 proteins were assigned to 25 different functional categories with 368 subsystems using SEED subsystems by RAST analysis (Figure S1). The annotated genome had 112 genes responsible for bacteriocins (2 genes), resistance to antibiotics and toxic compounds (84 genes), and invasion and intracellular resistance (26 genes; Figure S1).

The ANI and dDDH values of representative Flavobacterium species are summarized in Table 2. F. spartansii and Flavobacterium sp. WG21 have an ANI value of 96.9% and a dDDH value of 72.7% (Table 2), suggesting that they are the same species according to the microbial taxonomy for species delineation (95 and 70% cut-off for ANI and dDDH, respectively) (Goris et al., 2007).

**Gene Repertoire of Flavobacterium**
The genome size for ubiquitous genes (core genome) or entire set of genome (pan-genome) amongst the selected Flavobacterium genomes was plotted against the number of genomes (Figure 1). The pan-genome plot showed that the power trend line had not reached the plateau (Figure 1), indicating that Flavobacterium possesses an open pan-genome. Core genome analysis showed that the number of shared genes decreased with the addition of the input genomes and was predicted to converge against 1,182 (Figure 1). Actually, the core genome for the selected Flavobacterium in comparison was calculated to be 1,125 CDS per genome; thus, given the assumption that the prediction slightly overpredicts the core genome size, the current core genome represents the Flavobacterium genus quite well. F. spartansii shared at least 3,649, 3,201, 2,996, 2,774, 1,971, and 1,868 CDS with those environmental or opportunistic pathogenic Flavobacterium including Flavobacterium sp. WG21 (87.2% of its total encoding genes), F. hibernum DSM 12611 (70.1%), F. denitrificans DSM 15936 (71.7%), F. beibuense F44-8 (69.6%) F. daejeonense DSM 17708 (68.2%), and F. johnsoniae UW101 (61.6%) (Figure 2). On the other hand, it shared up to 1,627, 1,651, and 1,712 CDS with the selected pathogenic Flavobacterium species such as F. columnare strains (~81%) and three F. psychrophilum strains (~75%) and F. branchiophilum (~71.3%).

**Antimicrobial Resistance**
Antibiotic susceptibility assays were conducted using the disk diffusion method (Table 3). Of the 8 tested antibiotics, F. spartansii T16T was immediately sensitive to trimethoprim-sulfamethoxazole, polymyxin-B, florfenicol, erythromycin as well as oxytetracycline, while it was resistant to ampicillin, penicillin G, 2,4-diamino and 6,7-di-isopropyl pteridine (Table 3). The resistome in strain S12 was similar to that in strain T16T. However, compared to strain T16T, strain S12 was more sensitive to low concentrations of erythromycin and immediate sensitive to ampicillin. Neither of the two F. spartansii isolates (Table 3) were completely sensitive to the two antibiotics (i.e., florfenicol and oxytetracycline) that are approved to treat certain flavobacterial diseases (e.g., bacterial coldwater disease caused by F. psychrophilum, and columnaris disease caused by F. columnare) in food fish (Bowker et al., 2016).

Both the RAST SEED subsystem and CARD were used to identify antibiotic resistance genes within the F. spartansii genome using the default settings. The predicted genes conferring resistance to aminocoumarin, mupirocin, chloramphenicol, fluoroquinolone, tetracycline, rifampin, and β-lactam are summarized in Table S1. Interestingly, up to 10 β-lactam resistance-related genes were found in the F. spartansii T16T genome including those encoding the putative β-lactamases (8 copies), metallo-β-lactamases (1 copy) and penicillin-binding protein (1 copy). This observation is consistent with resistance to both penicillin and ampicillin in our antimicrobial tests (Table 3). Most β-lactamases in F. spartansii showed very low identity (<50%) to other fish pathogens (F. psychrophilum PG2, F. columnare and F. branchiophilum FL-15) (Table S1). However, a few lactamases have relatively high identity to those in F. johnsoniae and Flavobacterium sp. WG21. No erythromycin resistance related genes were detected in F. spartansii. More drug resistance genes and non-specific efflux pumps (5 hydrophobic/amphiphilic exporters, HAE1

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**Table 1** | The genome features of the selected Flavobacterium spp.

| Strains | Genome size (Mb) | CRISPR count | GC (%) | Predicted CDS | Total RNA | Enzyme count |
|---------|-----------------|---------------|--------|---------------|-----------|--------------|
| F. psychrophilum CSF259-93 | 2.90 | 1 | 32 | 2,520 | 198 | 650 |
| F. psychrophilum JIP02/86 | 2.86 | 1 | 33 | 2,432 | 73 | 649 |
| F. psychrophilum PG2 | 2.85 | 1 | 33 | 2,497 | 197 | 643 |
| F. columnare ATCC 49512 | 3.16 | 3 | 31 | 2,846 | 89 | 674 |
| F. columnare 94-081 | 3.30 | 1 | 31 | 2,847 | 89 | 705 |
| F. branchiophilum FL-15 | 3.56 | 3 | 33 | 3,034 | 53 | 730 |
| F. spartansii T16T | 5.35 | 0 | 36 | 4,422 | 130 | 905 |
| Flavobacterium sp. WG21 | 5.20 | 1 | 36 | 4,319 | 111 | 916 |
| F. hibernum DSM 12611 | 5.28 | 2 | 33 | 4,510 | 132 | 1,009 |
| F. daejeonense DSM 17708 | 4.20 | 0 | 35 | 3,622 | 89 | 873 |
| F. denitrificans DSM 15936 | 4.80 | 2 | 34 | 4,121 | 133 | 957 |
| F. johnsoniae UW101 | 6.10 | 1 | 34 | 5,017 | 82 | 1,003 |
| F. beibuense F44-8 | 3.80 | 0 | 38 | 3,454 | 44 | 720 |
### Table 2

Digital DNA-DNA Hybridization values (up, black font) and average nucleotide identity values (low, red font) amongst different *Flavobacterium* spp.

|                      | F. spartansii T16 | F. psychrophilum JIP02 | F. psychrophilum CSF259 | F. psychrophilum PG2 | F. beibuense F44-8 | F. daejeonense DSM 17708 | F. columnare ATCC 49512 | F. columnare 94-081 | F. columnare PH1 | F. johnsoniae UW101 | F. hibernum DSM 12611 | F. branchiophilum FL-15 | F. denitrificans DSM 15936 | Flavobacterium sp. WG21 |
|----------------------|-------------------|------------------------|-------------------------|---------------------|-------------------|-------------------------|--------------------------|---------------------|----------------|-------------------|------------------------|--------------------------|--------------------------|-------------------------|
| **F. spartansii T16** |                   |                        |                         |                     |                   |                         |                          |                     |                |                   |                         |                          |                          |                         |
| F. psychrophilum JIP02 | 21.4              | 78.39                  | 78.39                   | 78.39               | 98.9              | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. psychrophilum CSF259 | 21.2              | 78.39                  | 78.39                   | 78.39               | 99.51             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. psychrophilum PG2  | 21.4              | 78.44                  | 78.44                   | 78.44               | 99.51             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. beibuense F44-8    | 18.8              | 76.75                  | 76.75                   | 76.75               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. daejeonense DSM 17708 | 20.8              | 79.16                  | 79.16                   | 79.16               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. columnare ATCC 49512 | 23.2              | 78.52                  | 78.52                   | 78.52               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. columnare 94-081   | 21.6              | 77.74                  | 77.74                   | 77.74               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. columnare PH1      | 22.3              | 78.03                  | 78.03                   | 78.03               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. johnsoniae UW101   | 23.9              | 81.96                  | 81.96                   | 81.96               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. hibernum DSM 12611 | 25.5              | 81.47                  | 81.47                   | 81.47               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. branchiophilum FL-15 | 21.6              | 77.94                  | 77.94                   | 77.94               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| F. denitrificans DSM 15936 | 23.4              | 81.47                  | 81.47                   | 81.47               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
| Flavobacterium sp. WG21 | 72.7              | 88.92                  | 88.92                   | 88.92               | 78.39             | 99.51                   | 18.3                     | 78.39              | 78.39           | 99.51             | 99.51                  | 99.51                    | 99.51                    | 99.51                   |
family) possibly conferring antibiotic resistance are also conserved in *Flavobacterium* sp. WG21 and *F. hibernum* DSM 12611.

### Prophages

At least 5 prophages were predicted in *F. spartansii* by PHAST (Table S2). Among them, the prophage 1 (29.4 kb) consisted of 20 CDSs that encodes 12 proteins with known functions and 8 hypothetical proteins. Of interest, prophage 1 may be complete, as it consists of a phage tail, head, portal, integrase, lysin, terminase, and other component proteins involved in phage structure and assembly. The GC content (35.9%) in prophage 1 is very close to the average of GC in the whole genome (35.5%), indicating that prophage 1 may have been integrated into *F. spartansii* genome long ago. Unlike prophage 1, the remaining 5 prophages seem to be incomplete, as evidenced by the absence of a portion of phage structure proteins (Table S2). However, these regions carried genes encoding some important enzymes which may
contribute to flavobacterial fitness under certain conditions. For example, prophage 2 had a DNA polymerase III beta subunit, methylase, NinG recombination, ATPase and Rec T protein, indicating this region may participate in DNA replication and modification. Prophage 3 carried many genes encoding enzymes related to polysaccharide (capsule precursor) metabolism; there were at least two 1,3-N-acetylglucosaminyltransferases, four glycosyl transferases, one GDP-D-mannose 4,6-dehydratase, one alpha-1,2-fucosyltransferase, one fucose synthetase, one dTDP-d-glucose 4,6-dehydratase, one L-fucosamine transferase and one UDP-N-acetylglucosamine 2-epimerase (Table S2). Prophage 4 may be linked to RNA metabolism because ribokinase-like domain-containing protein, RNA polymerase, ribonuclease H and putative ribonuclease 3 were organized as a cluster. Prophage 5 had genes encoding arsenate reductase and metallophosphoesterase, which possibly contribute to heavy metal resistance.

**Genomic Islands and Conjugative Transposon (CTnFs)**

At least 8 genomic islands (GIs) were identified in *F. spartansii* (Figures 3A,B and Table S3) by both IslandPick and IslandPath-DIMOB methods (Nagamatsu et al., 2015). The GI size ranged from 11.3 to 63.8 Kb (Figure 3A). Genes encoding virulence factors, toxins, DNA metabolism, transposases, regulators, modification and restriction systems and resistance to antibiotics occurred in these GIs (Figure 3 and Table S3), indicating that *F. spartansii* T16T possibly acquired these genes, thereby forming GIs favoring adaptation to diverse environments (see below). Among the 8 GIs, GI-8 had the largest size (63.7 Kb). Notably, GI-8 has many genes encoding components of a conjugative transposon in Bacteroidetes (here, named CTnFs), e.g., traN, traM, traK, traf, tral, and trag. Moreover, the remaining components of this large conjugative transposon CTnFs can be found in GI-7 (33.3 kb) including traD, traC, traE, traF, traG, tral, traf, traK, traM, tral, and traq genes (Table S3). We further examined the surrounding regions around GI-7 and found the traA and traC genes which are located upstream of traD (Table 4). The distribution spectrum of the CTnFs-like conjugative transposons were investigated by searching the conserved transfer protein gene traG (a signature gene for conjugative transposons) among the selected *Flavobacterium* species. No traG gene sequences (cutoff 50%) were found in *F. psychrophilum, F. columnare*, and

**FIGURE 3 |** Genomic islands in *F. spartansii* T16T. **(A)** The 8 genomic islands were predicted by Islandviewer. **(B)** The schematic of GI-associated features. The relative locations of the 8 GIs were shown in the predicted genome. The conjugation protein genes belonging to “tra” and “GG” in CTnFs were centered in the GI-7 and GI-8 regions, respectively.
**TABLE 4 | Transfer proteins in the selected Flavobacterium spp.*.**

| Genome island (BHE19_RS) | Genotype | TraA | TraB | TraD | TraF | TraG | TraJ | TraK | TraM | TraN | TraO | TraQ |
|--------------------------|----------|------|------|------|------|------|------|------|------|------|------|------|
| GI-7                     | 20235    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20240    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20245    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20255    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20215    | 100  | 32   | 32   | 32   | -    | -    | -    | -    | -    | -    | 53   |
|                         | 20260    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20270    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20275    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20290    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20300    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 20305    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| GI-8                     | 17885    | 100  | 32   | 32   | 32   | -    | -    | -    | -    | -    | -    | 77   |
|                         | 17810    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 17825    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 17830    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 17840    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
|                         | 17845    | 100  | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |

*The numbers representing amino acid identity (%). "-" indicates that there is no hit output.

_F. branchiophilum_ FL-15. The same results were obtained when using other _tra_ genes to search against their genomes, indicating that the conjugative transposon is absent in these fish pathogens. By contrast, the similar conjugative transposons were observed in _F. johnsoniae_, _Flavobacterium_ sp. W21, _F. hibernum_ DSM 12611, _F. daejeonense_ DSM 17708, _F. denitrificans_ DSM 15936, and _F. beibuense_ F44-8 (Table 4).

GI-2 had many genes encoding respiratory chain components such as cytochrome C subunits, cytochrome P450, quinolcytochrome C oxidoreductase, and several other membrane proteins (Table S3). GI-2 had the predicted virulence factor _VirI_, possibly participating in the Type IV secretory pathway (Table S3). Immediately downstream of GI-2, there are genes involved in the gliding motility (_RemB_ and _SprA_). Another large genomic island, GI-6, contained a gene cluster encoding DNA repair proteins and enzymes such as MsrA (preventing oxidative damage), NTP pyrophosphohydrolases, chaperone protein DnaK, deoxyribodipyrimidine photolyase (DNA repair enzyme), DNA alkylation repair enzyme, peroxiredoxin, and DNA-damage-inducible protein D (Table S3). On the same GI, there were transcriptional regulators from GntR, HxlR, and AraC families, showing that the GI-1 may be involved in the defense against oxidative stress and DNA damage repair. Compared to the above 4 GIs, the remaining ones (GI-1, GI-3, GI-4, and GI-5) were smaller (Table S3).

**Prediction of Virulence Factors**

When the proteome of _F. spartansii_ T16^T_ was used in a BLAST search against MvirDB database, 1,001 virulence factors were predicted (cutoff E-10) (Table S4). Further, 133 putative virulence proteins were found to be highly conserved in _F. spartansii_ T16^T_ (cutoff 50%) (Table S4). Virulence factor matches in the MvirDB database include well-known proteases, catalase/peroxidase HPI, conjugal transfer proteins (4 _Tra_ components), molecular chaperones (GroEL, DnaJ, and DnaK), translation initiation factor IF-1, transcription regulation factors, antimicrobial resistance proteins, LPS biosynthesis protein WbpP, biopolymer transporter (ExbD and SusD/RagB), ribosomal proteins, carbon and nitrogen metabolism related proteins as well as iron-binding proteins (Table S4).

At least three hemolysin genes were present in _F. spartansii_ (Table 5), which complements observations that strain T16^T_ has _in vitro_ hemolytic activity (Figure 4). When grown on sheep’s blood agar plate for 48 h, colonies of _F. spartansii_ T16^T_ appeared greenish, suggesting _α_-hemolytic activity (Figure 4). Moreover, many genes encoding proteolytic enzymes were discovered in the genome of _F. spartansii_ T16^T_ (Table 5). Further, the amino sequence of a collagenase (BHE19_RS20565, peptidases U32) was conserved in most of the selected _Flavobacterium_ (Table 5).
TABLE 5 | Proteases and hemolysins in Flavobacterium spp.*.

| Locus (BHE19_RS) | Gene product | F. spartansii | T16T | F. hibernum DSM 12511 | F. johnsoniae UW101 | Flavobacterium sp. W2021 | F. psychrophilum JIP02/86 | F. psychrophilum FL-15 | F. columnare ATCC 49512 | F. psychrophilum CSP259-83 | F. psychrophilum PG2 | F. denitrificans DSM 15936 | F. beibuense F44-8 | F. daejeonense DSM 17708 | F. columnare 94-081 |
|------------------|--------------|--------------|------|------------------------|----------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| PROTEASES | | | | | | | | | | | | | | | |
| 04620 | Metalloprotease RseP | 100 | 86 | 86 | 99 | 43 | 41 | 41 | 43 | 43 | 69 | 67 | 79 | 42 |
| 16755 | Peptidase M1 | 100 | 88 | 90 | 99 | 47 | 45 | 44 | 47 | 47 | 91 | 46 | 67 | 44 |
| 22130 | Peptidase M43 | 100 | 85 | 85 | 100 | 58 | 65 | 67 | 58 | 58 | 84 | 35 | - | 68 |
| 09105 | Peptidase S8 | 100 | 78 | 72 | 99 | 55 | 49 | 51 | 55 | 55 | 84 | 35 | - | 68 |
| 04735 | Aminopeptidase | 100 | 78 | 72 | 99 | 55 | 49 | 51 | 55 | 55 | 84 | 35 | - | 68 |
| 20565 | Collagenase U32 | 100 | 78 | 72 | 99 | 55 | 49 | 51 | 55 | 55 | 84 | 35 | - | 68 |
| HEMOLYSINS | | | | | | | | | | | | | | | |
| 15730 | Hemolysin | 100 | 96 | 93 | 100 | 75 | 78 | 75 | 75 | 75 | 93 | 78 | 82 | 75 |
| 03430 | Hemolysin | 100 | 92 | 88 | 99 | 25 | 72 | 28 | 26 | 26 | 88 | 64 | 80 | 27 |
| 04015 | Hemolysin | 100 | 96 | 93 | 100 | 75 | 78 | 75 | 75 | 75 | 93 | 78 | 82 | 75 |

*The numbers representing amino acid identity (%). "-" indicates that there is no hit output.

Gliding Machinery and Random Mutation Analysis

In F. spartansii, at least 21 genes encoding proteins associated with gliding motility were identified, including gldA, gldB, gldC, gldD, gldE, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM, gldN, SprA, SprB, SprC, SprD, SprE, SprF, and SprT (Table 6). The motility protein sequences were conserved among these Flavobacterium genomes except GldE, SprB, and SprC (Table 6). Genes encoding PorV and RemA also existed in F. spartansii T16T (Table 6). Therefore, F. spartansii possesses the minimal requirement of the full function of T9SSs (also important for gliding) consisting of GldK, GldL, GldM, GldN, SprA, SprE, and SprT (McBride and Nakane, 2015).

A transposon used for random mutagenesis in flavobacteria, pHimarEm1, was functional in F. spartansii T16T. Out of 2,000 conjungetants, at least 4 mutants were recovered with impaired ability to spread on PY2 agar or glide on the surface of glass slides (Figure 5). For mutant 2F2-2, the transposon inserted 6-bp upstream of the ATG start codon of a gene encoding a hypothetical protein (BHE19_RS13930). For mutants 7B5-5, the insertion of pHimarEm1 transposon was 14 bp downstream of the ATG start codon of an aconitase gene (BHE19_RS00150). In mutant 10E3-2 the transposon inserted 1,380 bp downstream of the ATG start codon of the gldM gene (BHE19_RS21040). For the mutant M69, the transposon inserted at 67 bp downstream of the ATG start codon of EpsM gene (BHE19_RS15305). The difference in biomass determination was negligible between the WT and mutants, indicating that these gliding genes were not critical for cell growth in PY2 broth (Figure 5).

DISCUSSION

Our results demonstrated that strain T16T possesses some virulence factors that are similar to those previously reported in other Bacteroidetes, including metalloproteases, and hemolysins (Table 5). Moreover, the type 9 secretion system (T9SS) also occurs in T16T, which may contribute to secreting proteins that may be involved in virulence (Table 6). Nevertheless, our results also show that T16T has some virulence factors and antimicrobial genes absent in well-known fish pathogens (i.e., F. columnare, F. Branchiophiilum, and F. psychrophilum; Table 5). The GC content in strain T16T (36%) was higher than in F. columnare ATCC 49512 (31%), F. Branchiophiilum FL-15 (33%), and F. psychrophilum JIP02/86 (33%), indicating their diverse genome evolution under differential selection (Lassalle et al.,...
TABLE 6 | Comparison of gliding machinery in selected *Flavobacterium* spp.

| Gene product | Locus (BHE19_RS) | F. spartansii T16T | F. psychrophilum JIP02/86 | F. psychrophilum CSF35943 | F. psychrophilum PG2 | F. beibuense F44-8 | F. daejeonense DSM 17708 | F. columnare ATCC 49512 | F. johnsoniae 94-081 | F. hibernum DSM 12611 | F. branchiophilum FL-15 | F. denitrificans DSM 15938 | Flavobacterium sp. W22 | *, ** indicates that there is no hit output.
|--------------|-----------------|------------------|---------------------|---------------------|-----------------|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| **GENE PRODUCTS INVOLVED IN GLIDING** | | | | | | | | | | | | | | |
| gldA | 02885 | 100 | 77 | 77 | 77 | 77 | 83 | 73 | 73 | 92 | 94 | 77 | 93 | 99 |
| gldB | 20835 | 100 | 55 | 55 | 55 | 49 | 65 | 49 | 50 | 88 | 89 | 57 | 88 | 99 |
| gldC | 20840 | 100 | 83 | 83 | 83 | 76 | 85 | 85 | 79 | 79 | 93 | 96 | 82 | 94 | 100 |
| gldD | 02790 | 100 | 59 | 59 | 59 | 57 | 49 | 50 | 88 | 89 | 57 | 88 | 99 |
| gldE | 02795 | 100 | 23 | 23 | 23 | 26 | 63 | 26 | 26 | 90 | 90 | 29 | 88 | 100 |
| gldF | 01675 | 100 | 75 | 75 | 75 | 76 | 81 | 76 | 76 | 96 | 94 | 72 | 96 | 100 |
| gldG | 01670 | 100 | 61 | 61 | 61 | 61 | 77 | 59 | 58 | 88 | 92 | 61 | 87 | 99 |
| gldH | 05785 | 100 | 61 | 61 | 61 | 61 | 77 | 59 | 58 | 88 | 92 | 61 | 87 | 99 |
| glia | 02925 | 100 | 56 | 56 | 56 | 57 | 65 | 53 | 52 | 80 | 84 | 57 | 76 | 99 |
| glia | 02660 | 100 | 70 | 70 | 70 | 68 | 79 | 69 | 68 | 94 | 96 | 73 | 92 | 100 |
| gldK | 21030 | 100 | 76 | 76 | 76 | 76 | 86 | 76 | 76 | 96 | 97 | 80 | 97 | 100 |
| gldL | 21035 | 100 | 87 | 87 | 87 | 87 | 74 | 73 | 73 | 94 | 89 | 72 | 96 | 99 |
| gldM | 21040 | 100 | 68 | 68 | 68 | 68 | 51 | 79 | 65 | 65 | 94 | 86 | 63 | 88 | 100 |
| gldN | 21050 | 100 | 64 | 64 | 64 | 64 | 50 | 70 | 63 | 62 | 88 | 88 | 61 | 87 | 99 |
| **GENE PRODUCTS INVOLVED IN SPREADING** | | | | | | | | | | | | | | |
| sprA | 00945 | 100 | 64 | 64 | 64 | 60 | 72 | 57 | 58 | 88 | 91 | 60 | 88 | 99 |
| sprB | 05460 | 100 | 39 | 37 | 39 | 40 | 38 | 33 | 39 | 37 | 37 | 37 | 98 |
| sprC | 05450 | 100 | 36 | 36 | 36 | 34 | 43 | 34 | 32 | 34 | 34 | 60 | 99 |
| sprD | 05455 | 100 | 43 | 43 | 43 | 43 | 55 | 42 | 39 | 80 | 83 | - | 82 | 99 |
| sprE | 05120 | 100 | 49 | 49 | 49 | 49 | 55 | 48 | 47 | 81 | 83 | 45 | 78 | 99 |
| sprF | 05455 | 100 | 43 | 43 | 43 | 43 | 55 | 42 | 39 | 80 | 83 | - | 82 | 99 |
| sprT | 03165 | 100 | 57 | 57 | 57 | 57 | 67 | 56 | 59 | 88 | 92 | 61 | 87 | 100 |
| **OTHERS INVOLVED IN T9SS SECRETION** | | | | | | | | | | | | | | |
| porV | 02670 | 100 | 68 | 68 | 68 | 68 | 71 | 64 | 66 | 87 | 92 | 65 | 83 | 99 |
| remA | 22245 | 100 | 40 | 40 | 40 | 42 | 50 | 31 | 41 | 48 | 74 | 36 | 48 | 83 |

Among the selected flavobacteria, the genome size of *F. spartansii* T16T (5.35 Mb, next to *F. johnsoniae*) was remarkably larger than that in typical fish pathogens such as *F. columnare* ATCC 49512 (3.16 Mb), *F. branchiophilum* (3.56 Mb), and *F. psychrophilum* JIP02/86 (2.86 Mb). Genome size in opportunistic pathogens is typically larger than in virulent strains because more functional genes are required for their complicated living styles and diverse environments. Kolton et al. (2013) reported the genome size in *Flavobacterium* belonging to the terrestrial clade is around 40% larger than within the aquatic clade (Kolton et al., 2013).

Genomic islands often drive microbial evolution, increase fitness, and contribute to species diversity (Jackson et al., 2011). It is interesting that a large conjugative transposon (CTnFs) spanning at least two large GIs (GI-7 and GI-8) was found in *F. spartansii* T16T. Such conjugative transposons are widespread in *Bacteroides*, *Prevotella*, and *Porphyromonas* (Franco, 2004; Naito et al., 2011; Gorenc et al., 2012). Here, we extended the distribution profiles of conjugative transposons to some flavobacterial members including *F. spartansii*, *F. johnsoniae*, *F. hibernum*, *F. denitrificans*, *F. beibuense*, and *F. daejeonense* (Table 4). Gene content and organization of CTnFs-like conjugative transposons in *F. spartansii* mimic those reported in transposons CTn3-Bf and CTnB14 of *B. fragilis* YCH46 and *P. bryantii* B14T (Gorenc et al., 2012). One of the most intriguing features of conjugative transposon is presence of two conserved regions called “tra” and “GG” (Gorenc et al., 2012). However, CTnFs genes have not been reported in *F. columnare*, *F. branchiophilum* and *F. psychrophilum* species based on marker gene search (traG), nor were CTnFs genes detected in *Flavobacterium* sp. W22 (Table 4) though its phylogeny placement is very close to *F. spartansii*. 

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T16<sup>T</sup>. The reason for the absence of CTnFs-like transposons in typical *Flavobacterium* pathogens remains unknown. The size of conjugative transposon is generally large (100–210 kb in *Bacteroides*) but varies considerably amongst species. CTnB<sub>t</sub>4 in *P. bryantii* B<sub>t</sub>4T is around 212 kb with a spacer (49.1 kb) where a large sugar utilization gene cluster locates (Gorenc et al., 2012). This conjugative transposon may promote the ability to scavenge the glycans in vivo in this oral cavity-adapted symbiont (Matsui et al., 2000). The accurate size, full contents and physiological roles in the CTnFs remain unclear in free-living isolates or opportunistic pathogens (see next). However, many functional gene products were predicted between/around the two regions (Gorenc et al., 2012). While the conjugative transposon is activated and mobilized, it frequently carries over its close regions to the new hosts (Juhas et al., 2009). Thus, the conjugative transposons may have important implications for spreading virulence factors, metabolism genes and some resistance genes (Burrus and Waldor, 2004; Juhas et al., 2009). Furthermore, the transfer efficiency of CTnDOT/ERL conjugative transposons in *Bacteroides* (also found in other Bacteroidetes members such as *Prevotella*), improved at least 1,000-fold when a low concentration of tetracycline was present in the culture (Jeters et al., 2009). The phenomenon indicates that antibiotics in the environment (such tetracycline) not only provide selection pressure for Bacteroidetes to acquire foreign antimicrobial genes, but also promote spreading other antibiotic genes (e.g., erythromycin gene transfer in *Bacteroides*) or other genetic elements (Shoemaker et al., 2001). Because the conjugative transposons present in *Flavobacterium* strains are very similar to CTnDOT, one can infer that horizontal transfer of flavobacterial genes involved in antibiotic resistance and/or virulence factors is very likely in their hosts and native habitats.

There are multiple antibiotics and chemotherapeutic compounds that are currently approved for use in foodfish or can be utilized under an investigational new animal drug (INAD) status (https://www.fws.gov/fisheries/aadap/aquaculture.html). Thus, generating more antibiotic sensitivity data for *F. spartansii* is crucial for guiding further research toward additional antibiotic approvals. *F. spartansii* T16<sup>T</sup> is intermediate in susceptibility to the permitted antibiotics in the USA aquaculture industry, namely oxytetracycline, sulfadimethoxine and florfenicol. One of the striking features in resistome analysis of T16<sup>T</sup> is that there are at least 10 β-lactam resistance-related genes. Putative β-lactamases (8 copies), metallo-β-lactamases (1 copy) and penicillin-binding proteins (1 copy) possibly confer resistance to penicillin and ampicillin (Table S1). Resistance toward third-generation cephalosporins (data not shown) by class C beta-lactamases seen in *F. spartansii* would hamper treatment of infection by *Flavobacterium* species. The concern is that, as a reservoir of extended-spectrum class C beta-lactamases, *F. spartansii* may spread these resistance gene(s) to other flavobacterial pathogens in the future. Extensive antibiotics application in the aquaculture industry may account for the multidrug resistance profile seen in resistance genes of *F. spartansii* (Kristiansson et al., 2011). It is interesting that two genes conferring tetracycline resistance were found in *F. spartansii* (as in several other *Flavobacterium* species) while disc analysis showed that bacterial cells were intermediate in susceptibility to tetracycline (Table 3). A tetracycline resistance (*tetX*) gene in transposons Tn4351 and Tn4400 did not confer resistance on anaerobically-grown *Bacteroides fragilis* while it functioned in aerobically-grown *E. coli* (Speer and Salyers, 1990; Speer et al., 1991). Gene *tetX*, located inside the conjugative transposon, may be acquired from other bacteria (exemplified by the GC ratio compared to the average ratio in the genome) during the transposon mobilization process. Expression of *tetX* may be problematic in *F. spartansii* because it is known that the transcriptional and translational initiation signals from Proteobacteria or the Gram-positive bacteria are not recognized well in Bacteroidetes (Chen et al., 2007a). Drug resistance mechanisms in bacteria are multifactorial, and can involve enzymatic degradation of the drugs, direct extrusion of the drug from the cells through efflux pumps, or alteration/mutation of ribosomal binding sites (Nikaido, 2009). The multidrug resistance efflux pumps predicted in *F. spartansii* and other flavobacteria are possibly involved in antimicrobial resistance, which does not manifest in other antibiotic degradation genes (Nikaido, 2009; Sun et al., 2014). This situation is cause for concern, and warrants more stringent surveillance in the use of antibiotics, as well as the resultant antibiotic resistance in clinically important bacterial species. Whole genome sequence of *F. spartansii* will be useful in future studies to determine antimicrobial resistance and virulence attributes as well as mechanisms that enhance its environmental or host fitness.

Besides the conjugative transposons and genomic islands, bacteriophages and phage-like genetic elements occupy a great amount of bacterial genome space. Bacteriophage-mediated transduction is an important contributor to spreading antimicrobial resistance and metabolism genes by the horizontal gene transfer mechanisms (Dutta and Sarkar, 2015). At least
5 prophages were predicted in *F. spartansii* T16\(^T\) (Table S2). Only one of them (prophage 1) seems to be complete. However, the other prophages should not just be regarded as inactive because they may share machinery (e.g., structure proteins or replication enzymes) originating from prophage 1 or from the host, thus they can successfully assemble, pack and activate (Drulis-Kawa et al., 2012). Genes encoding important enzymes involved in capsule precursor biosynthesis and heavy metal resistance were found within the regions of the predicted prophage genomes, indicating that it is possible that these prophages possibly shape the bacterial genome evolution (Table S2). However, how these prophages influence the host behavior and virulence remains unexplored in flavobacteria. Indeed, prophages or phage-like genetic elements are not as prevalent in flavobacterial pathogen genomes as those in *F. spartansii* T16\(^T\); e.g., most of *F. psychrophilum* strains (such as 950106-1/1, JIP02/86, MH1, PG2, and 5) carry only one prophage (named 6H); moreover, there are only 1 and 3 incomplete phage clusters (no complete one) identified in *F. columnare* 94-081 and *F. columnare* ATCC 49512, respectively. Similarly, Touchon et al. reported that there were no incomplete prophage elements in *F. branchiophilum* FL-15 (Touchon et al., 2011). This may be partially explained due to the presence of multiple CRISPR loci in *F. psychrophilum* (1–2 loci) (Castillo et al., 2016), *F. columnare* (at least 3 loci) (Kayansamruaj et al., 2017), and *F. branchiophilum* FL-15 (at least 3 loci) (Touchon et al., 2011; Barrangou and Marraffini, 2014; van Houte et al., 2016). It is interesting that we did not detect any CRISPR elements in *F. spartansii* T16\(^T\), which may coincide with presence of prophages, genomic islands, and conjugative transposons in its genome. CRISPR/Cas, the prokaryotic immune system, defends the foreign DNA invasion (plasmids, phages and transposons) and may participate in regulating stress gene response and controlling bacterial virulence (Louwen et al., 2014; Laanto et al., 2017).

Prediction of virulence factors in this study contributes to our understanding of flavobacterial pathogenesis mechanisms as well as Flavobacterium/host interactions. The predicted virulence factors have good homology with those discovered in other well-known pathogenic flavobacteria (Touchon et al., 2011; Kumru et al., 2017). For example, *F. spartansii* T16\(^T\) has the capability to digest animal erythrocytes with hemolytic activities, consistent with three hemolysins genes predicted in *F. spartansii* as virulence factors (Table 5). Hemolysins are well-documented cytolytic toxins that are important for animal pathogenesis process such as sepsis and tissue damage (Portnoy et al., 1988; Los et al., 2013). In *F. columnare* strain 94-081, disruption of one of the hemolysin genes (AWN65_RS11020) decreased fish mortality 15%, indicating that it contributes to virulence (Kumru et al., 2017). Moreover, this observation also showed that additional hemolysin(s) may be necessary to have full virulence against fish (Kumru et al., 2017). In addition, *F. spartansii* T16\(^T\) has the thiol-activated cytolyisin (TACYS) that possibly forms pores on the erythrocyte membrane (Morgan et al., 1996). Moreover, TACYS can also lead to the triggering signaling pathways in host cells, as is the case for listeriolysin O (LLO) (Hamon et al., 2012). LLOs induce a cytokine response by functioning as a pleiotropic pseudocytokine/chemokine, thus strongly influencing the course of infection (Baba et al., 2002). Protein sequences of a collagenase (peptidases U32) in strain T16\(^T\) are conserved amongst the selected *Flavobacterium* strains (Table 5). Nakayama et al. (2016) found that the collagenase gene (*fpcol*) contributed to mortality in the Ayu (*Plecoglossus altivelis*) (Nakayama et al., 2016). Further, they also reported that the expression of *fpcol* was partially repressed by calcium or gelatin, showing that collagenase was necessary when the bacterial cells adhered to fish surface and initiated the invasion process (Nakayama et al., 2016). *F. spartansii* T16\(^T\) has several metalloproteases (Table 5), which may facilitate bacterial dispersion and tissue damage. Conjugal transfer proteins (4 Tra components) as virulence factors probably allow these genomic elements to transfer from or to other bacteria (Vogel et al., 1998).

Fourteen *gld* and seven *spr* genes participating in the gliding motility, respectively, were found in *F. spartansii* T16\(^T\) genome (Table 6). The amino acid sequences of the gliding and spreading proteins are highly conserved among the selected flavobacteria except *GldE* (Table 6). *GldE* (cutoff 50%) seem to be absent in the genomes of *F. psychrophilum* and *F. columnare*, indicating that they are not critical for gliding in these two *Flavobacterium* species. In *F. johnsoniae*, the gliding function of *gldE* can be replaced by *gldB* (Hunnicutt and McBride, 2001). Seven spreading protein encoding genes (SprA, SprB, SprC, SprD, SprE, SprF, and SprT) were found in *F. spartansii* T16\(^T\) genome (Table 6). Most of the gene products (except SprB) show good homology to those in *F. johnsoniae* and *F. hibernum* DSM 12611, *F. denitrificans* DSM 15936 and *Flavobacterium* sp. WG21 though some of them (SprB and SprC) have the relative low identity to those in fish pathogens *F. psychrophilum* and *F. columnare* (Table 6).

Genetic amenability of *F. spartansii* T16\(^T\) was exemplified by obtaining several gliding-deficient mutants using a transposon mutagenesis method. It is not surprising that we found one of the motility mutants had disruption of the gliding gene *gldM*. Disability of *gldM* gene expression in *F. johnsoniae* and *Cellulophaga algicola* caused gliding deficiency on the slide or agar surfaces, as well as failure of the delivery of SprB to the cell surface, and secretion of many cell surface and extracellular proteins (Braun et al., 2005; Zhu and McBride, 2016). Moreover, disruption of some gliding genes in flavobacteria led to attenuated virulence or biofilm formation (Álvarez et al., 2006; Sato et al., 2010). Decreasing virulence in gliding mutants may be due to the disruption of the T9SSs function (Sato et al., 2010). Diverse CTDs involved in protein secretion were recently characterized in *F. johnsoniae* (Chen et al., 2007b; Kulkarni et al., 2017). Proteins secreted through T9SSs possess two critical signals: the first one is the N-terminal signal peptides that are required by the Sec system to export outside the cytoplasmic membrane; and the second one is the carboxy-terminal domains (CTDs) that are needed by the T9SSs to secrete through outer membranes (McBride and Zhu, 2013; de Diego et al., 2016; Kulkarni et al., 2017). However, more research is needed to provide more evidences for virulence factors secreted through T9SSs in these flavobacterial pathogens. Furthermore, three putative gliding motility genes encoding the hypothetical protein...
belonging to the HCP-like family, the aniconase involved in TCA cycle for energy production, and a polysaccharide synthesis component. The complementation of these mutants is warranted to further confirm the gene function(s) involved in gliding motility.

AUTHOR CONTRIBUTIONS

SC and EW conceived the study and participated in its design and coordination. SC performed the experiments, whole genome sequencing, annotation, and comparative analysis. JB contributed to the genome analysis. SC, TL, MF, and EW wrote the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02339/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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