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Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum* ATCC 49418^T

Anson KK Wu[1], Andrew M Kropinski[1], John S Lumsden[1], Brian Dixon[2] and Janet I MacInnes[1*]

Abstract

Flavobacterium psychrophilum is the causative agent of bacterial cold water disease and rainbow trout fry mortality syndrome in salmonid fishes and is associated with significant losses in the aquaculture industry. The virulence factors and molecular mechanisms of pathogenesis of *F. psychrophilum* are poorly understood. Moreover, at the present time, there are no effective vaccines and control using antimicrobial agents is problematic due to growing antimicrobial resistance and the fact that sick fish don't eat. In the hopes of identifying vaccine and therapeutic targets, we sequenced the genome of the type strain ATCC 49418 which was isolated from the kidney of a Coho salmon (*Oncorhychus kisutch*) in Washington State (U.S.A.) in 1989. The genome is 2,715,909 bp with a G+C content of 32.75%. It contains 6 rRNA operons, 49 tRNA genes, and is predicted to encode 2,329 proteins.

Keywords: Aerobic, Gram negative, Psychrotolerant, Fish pathogen, *Flavobacterium*, Bacterial cold water disease, Rainbow trout fry mortality syndrome

Introduction

Flavobacterium psychrophilum is a Gram-negative pathogen that infects all species of salmonid fish and has been found to also infect eel and three species of *cyprinids* [1-3]. It causes bacterial cold water disease (BCWD) and rainbow trout fry mortality syndrome (RTFS) in fish and is responsible for significant losses in the salmonid aquaculture industry [1]. Water temperature plays a key role in the infection and development of disease [4] which occurs between 4-16°C and is most prevalent at 10°C or below [5]. It was originally thought to be limited to North America [6] but it is now recognized in almost every country in Europe, in some parts of Asia, and in Australia [1,7].

Three serotypes and two biovars of *F. psychrophilum* have been described [7,8]. In addition, molecular analysis of the population structure of this bacterium suggests that there are a number of distinct lineages [7]. It has been speculated that some strains are species specific [9] while others are location specific [10]. Some strains have also been observed to cause only either BCWD or RTFS [7]. A recent study in Japan showed multiple sequence types infecting ayu (*Plecoglossus altivelis*) in a closed lake environment [11]. It is also known that phase variation can occur where the colonial phenotype changes between "rough" and "smooth", perhaps to help in evasion of the immune system [12]. Generally *F. psychrophilum* populations are heterogeneous; however, a recent study showed closely related epidemic clones infecting rainbow trout (*Oncorhynchus mykiss*) in Nordic countries [13]. To date, only one genome sequence [14] of *F. psychrophilum* has been reported and sequences of other strains are required to gain insight into the molecular mechanisms of virulence and why some strains are more virulent than others. Here we present a summary of classification and features of the *F. psychrophilum* type strain ATCC 49418 (= DSM 3660 = NCMB = 1947 = LMG 13179 = ATCC 49418) [15] together with a description of the complete genome and its annotation.

Organism Information

Classification and Features

The taxonomy of *F. psychrophilum* has been changed many times since Borg (1960) classified it as *Cytophaga psychrophila* based on its biochemical properties [16]. It was later reclassified within the genus *Flexibacter* based on DNA homology and renamed to *Flexibacter psychrophilus* [17]. Most recently, it was reclassified to the genus

* Correspondence: macinnes@uoguelph.ca
[1]Department of Pathobiology, Ontario Veterinary College, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada
Full list of author information is available at the end of the article

Table 1 Classification and general features of *Flavobacterium psychrophilum* ATCC 49418^T

MIGS ID	Property	Term	Evidence code[a]
	Current Classification	Domain *Bacteria*	TAS [24]
		Phylum *Bacteroidetes*	TAS [25]
		Class *Flavobacteriia*	TAS [26,27]
		Order *Flavobacteriales*	TAS [23]
		Family *Flavobacteriacea*	TAS [18,22]
		Genus *Flavobacterium*	TAS [18,28]
		Species *Flavobacterium psychrophilum*	TAS [18]
		Type strain ATCC 49418	TAS [15,18]
	Gram stain	Negative	TAS [15]
	Cell shape	Rods	TAS [15]
	Motility	Gliding	TAS [15]
	Sporulation	Non-spore forming	TAS [18]
	Temperature range	Psychrotolerant (4°C to 30°C)	TAS [15,29,30]
	Optimum temperature	15-20°C	TAS [31,32]
	Carbon source	Non-saccharolytic	TAS [18]
	Energy source	Chemoorganotroph	TAS [18]
	Terminal electron receptor	Oxygen	NAS [33]
MIGS-6	Habitat	Host	TAS [15]
MIGS-6.3	Salinity	Usually grows in 0.5% and stops at 1.0%	TAS [8,15]
MIGS-22	Oxygen	Aerobic	TAS [15]
MIGS-15	Biotic relationship	Obligate pathogen of fish (but can survive in freshwater for several months)	NAS [7]
MIGS-14	Pathogenicity	Salmonid fishes, eel, and three species of *Cyprinids*	TAS [1,15]
MIGS-4	Geographic location	Worldwide including North America, Europe, and Asia	TAS [1,7]
MIGS-5	Sample collection time	1989	TAS [15]
MIGS-4.1 MIGS-4.2	Latitude – Longitude	Not reported	
MIGS-4.3	Depth	Not Reported	
MIGS-4.4	Altitude	Not Reported	

[a]Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from of the Gene Ontology project [40].

Flavobacterium and renamed to *F. psychrophilum* based on DNA-RNA hybridization [18]. The genus name was derived from the Latin *flavus* meaning "yellow" and the ancient Greek *βακτήριον* (baktḗrion) meaning "a small rod" giving the Neo-Latin word *Flavobacterium*, a "small yellow rod-shaped bacteria" [19,20]. The species name was derived from the Greek word *psuchros* (ψυχρός) meaning "cold" and the Neo-Latin word *philum* meaning "loving" which translates to "cold loving" [19,20]. The genus *Flavobacterium* consists of 119 recognized species [21]; it belongs to the family

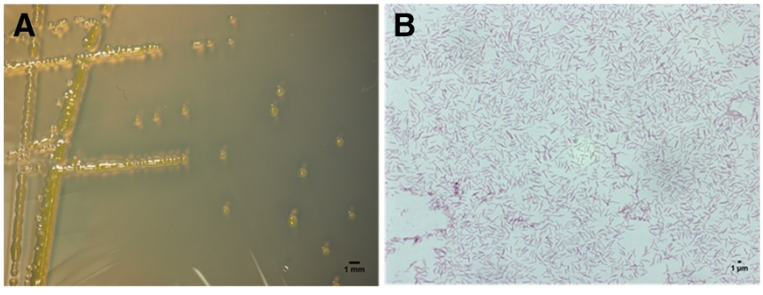

Figure 1 Colonial and cellular morphology of *F. psychrophilum* ATCC 49418^T grown on cytophaga agar (A) and Gram stained (B) (1000x).

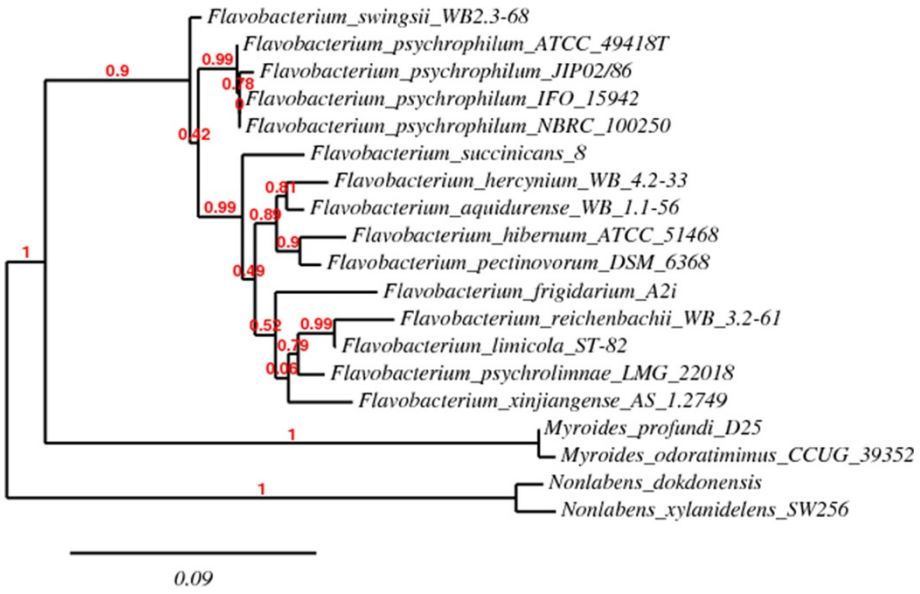

Figure 2 Phylogenetic tree displaying the relationship between *F. psychrophilum* ATCC 49418^T and selected strains and species of the same genus. Other genera from the family *Flavobacteriaceae* were used as an out group. The phylogenetic tree was constructed using the "One Click" mode with default settings in the Phylogeny.fr platform [41]. This pipeline uses four different programs including MUSCLE [42], Gblock [43], PhyML [44], and TreeDyn [45]. The numbers above the branches are tree support values generated by PhyML using the aLRT statistical test.

Flavobacteriaceae [18,22] and the order *Flavobacteriales* [23] (Table 1).

F. psychrophilum ATCC 49418^T was isolated in Washington State (U.S.A) from the kidney of a young Coho salmon (*Oncorhynchus kisutch*) in 1989 [15]. It is a Gram negative, aerobic, and psychrotolerant microorganism [7] (Figure 1). When grown on cytophaga agar, bright yellow, smooth, discreet, circular, convex, and non-adherent colonies are produced [8]. The optimal growth temperature is between 15-20°C [31,32] with no growth occurring at 30°C or greater [15,29,30]. Microscopically it is rod-shaped measuring 3–7 μm long and 0.3-0.5 μm wide [8]. Although gliding motility has been reported the mechanism is yet to be elucidated since *F. psychrophilum* does not appear to use pili or polysaccharide secretion [1,15,17]. API-ZYM tests show that it can produce alkaline phosphatase, esterase, lipase, leucine, valine, and cysteine arylamidases, trypsin, acid phosphatase, and napthol-AS-BI phosphohydrolase [8]. In addition, it has been reported that it can produce catalase [29,34] and oxidase [17], hydrolyze tributyrin and proteins including casein, gelatin, elastin, albumin, collagen, and fibrinogen [35-39]. Although many strains including ATTC 49418^T cannot metabolize simple and complex sugars [1] a recent study has shown that some strains are able to produce two or more sugar degrading enzymes including alpha-galactosidase, beta-galactosidase, alpha-glucosidase, beta-glucosidase, and N-acetyl-beta-glucosaminidase [8].

A phylogentic tree was constructed using the 16S rRNA sequences of *F. psychrophilum* ATCC 49418^T, selected strains and species of the same genus, as well as selected species of other genera belonging to the family *Flavobacteriaceae* (Figure 2). The four *F. psychrophilum* strains are grouped together in the tree with ATCC

Table 2 Project information

MIGS ID	Property	Term
MIGS-31	Finishing quality	Finished
MIGS-28	Libraries used	None
MIGS-29	Sequencing platforms	PacBio RS II
MIGS-31.2	Fold coverage	184x
MIGS-30	Assemblers	HGAP workflow
MIGS-32	Gene calling method	NCBI Prokaryotic Genome Annotation Pipeline, GeneMarkS+
	Locus Tag	FPG3
	GenBank ID	CP007207
	GenBank Date of Release	September 12, 2014
	BioProject ID	PRJNA236029
	GOLD ID	Gi0074339
	Project relevance	Fish Pathogen
MIGS-13	Source Material Identifier	ATCC

49418^T being most similar to JIP02/86 (ATCC 49511), the only other strain to have a complete genome sequence.

Genome sequencing information

Genome project history

The complete genome sequence and annotation data of *F. psychrophilum* ATCC 49418^T have been deposited in DDBJ/EMBL/GenBank under the accession number CP007207. Sequencing and assembly steps as well as finishing were performed at McGill University and Génome Québec Innovation Centre. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline [46] and manually edited in Kodon (Applied Maths, Austin, TX). Table 2 presents a summary of the project information and its association with MIGS version 2.0 compliance [47].

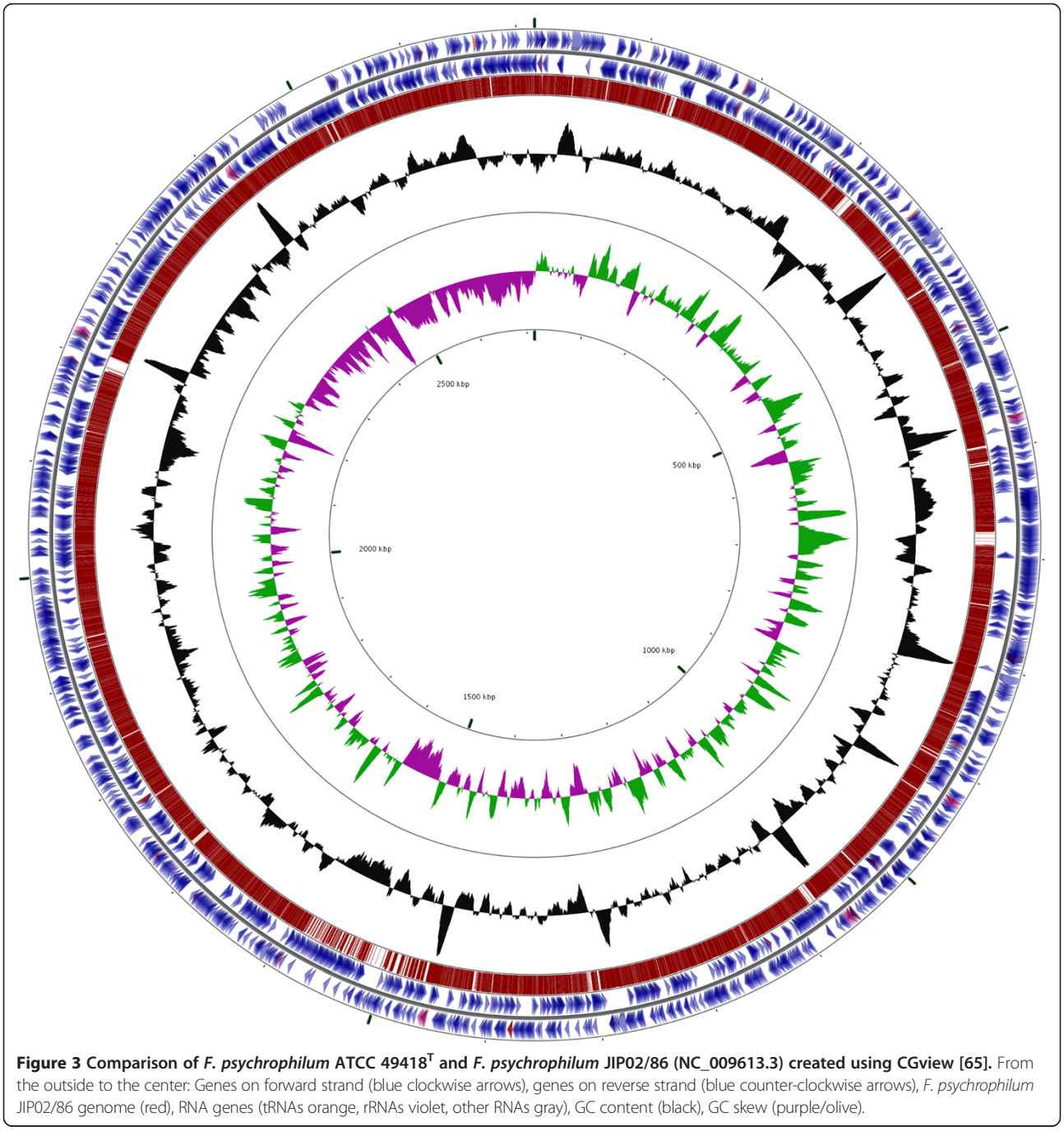

Figure 3 Comparison of *F. psychrophilum* ATCC 49418^T and *F. psychrophilum* JIP02/86 (NC_009613.3) created using CGview [65]. From the outside to the center: Genes on forward strand (blue clockwise arrows), genes on reverse strand (blue counter-clockwise arrows), *F. psychrophilum* JIP02/86 genome (red), RNA genes (tRNAs orange, rRNAs violet, other RNAs gray), GC content (black), GC skew (purple/olive).

Growth conditions and DNA isolation

F. psychrophilum ATCC 49418^T was originally obtained from the American Type Culture Collection [15] and was stored in a frozen glycerol stock (15%) at −70°C. It was grown for 4 days at 12°C on modified cytophaga agar [48] containing 0.06% (*w/v*) tryptone, 0.05% yeast extract, 0.02% beef extract, 0.02% sodium acetate, 0.05% anhydrous calcium chloride, 0.05% magnesium chloride, 0.05% potassium chloride, 1.5% agar, 0.02% gelatin, pH 7.5. Well isolated colonies were used for genomic DNA isolation. Colonies (~ 4 mm^3) were picked using a sterile toothpick and lysed using modified B1 (1 50 mM Tris·Cl, 50 mM EDTA, 0.5% Tween®-20, 0.5% Triton X-100, pH 8.0) and B2 (750 mM NaCl, 50 mM MOPS, 15% isopropanol, 0.15% Triton X-100, pH 7.0) buffers. DNA was purified and eluted using the QIAGEN Plasmid Midi Kit (Qiagen, Germany) following manufacturer's protocol.

Genome sequencing and assembly

Genome sequencing of *F. psychrophilum* ATCC 49418^T was performed using a PacBio RS II instrument. The reads were automatically processed through the Single Molecule Real Time (SMRT) software suite using the Hierarchical Genome Assembly Processing (HGAP) pipeline [49]. The resulting reads (580,625,890 bp in total) were filtered and the longest reads with 20x coverage were selected as seeds for constructing preassemblies. The preassemblies were constructed by aligning the short reads to the long reads (seeds). Each read was mapped to multiple seeds using BLASTR [50]. In total there were 8073 long sequences totaling 90,000,401 bp with an average length of 11148 bp and 162,858 bp short sequences totaling 490,625,489 bp with an average length of 3013 bp. Since errors in PacBio are random, aligning the multiple short reads onto the long reads allows the correction of errors in the long reads. The optimal number of sequences to be mapped onto the seeds is controlled by the "-bestn" parameter and the optimal number was determined to be 12. The preassembled reads for the seeds are generated using PBDAG-Con [51] to create corrected consensus sequences in addition to quality analysis of the seeds. This script uses multiple sequence alignments and a directed acyclic graph to produce the best consensus reads possible. It does so by eliminating the insertion and deletion errors generated during the sequencing process. In addition, it avoids generating chimeric sequences (sequences with artifacts) for

Table 3 Nucleotide content and gene count levels of the genome

Attribute	Genome (total)	
	Value	% of total
Genome size (bp)	2,715,909	100.00%
DNA coding (bp)	2,336,075	86.01%
G+C content (bp)	889,460	32.75%
DNA scaffolds	1	
Total genes	2397	100.00%
Protein-coding genes	2,329	97.00%
RNA genes	68	2.84%
Pseudo genes	24	1.00%
Genes in internal clusters	N/D[a]	
Genes with function prediction	1881	78.47%
Genes assigned to COGs	1,438	60.00%
Genes assigned Pfam domains	1,933	80.64%
Genes with signal peptides	236	9.85%
Genes with transmembrane helices	506	21.11%
Number of CRISPR candidates	8	
Confirmed CRISPR(s)	1	
Unconfirmed CRISPR(s)	7	

[a]N/D = not determined.

Table 4 Number of genes associated with the 25 general COG functional categories

Code	Value	% of total	Description
J	140.0	9.66	Translation
A	0.0	0.00	RNA processing and modification
K	72.0	4.97	Transcription
L	96.0	6.63	Replication, recombination and repair
B	0.0	0.00	Chromatin structure and dynamics
D	18.0	1.24	Cell cycle control, mitosis and meiosis
Y	0.0	0.00	Nuclear structure
V	41.0	2.83	Defense mechanisms
T	32.0	2.21	Signal transduction mechanisms
M	145.0	10.01	Cell wall/membrane biogenesis
N	4.0	0.28	Cell motility
Z	1.0	0.07	Cytoskeleton
W	0.0	0.00	Extracellular structures
U	31.0	2.14	Intracellular trafficking and secretion
O	61.0	4.21	Posttranslational modification, protein turnover, chaperones
C	75.0	5.16	Energy production and conversion
G	51.0	3.52	Carbohydrate transport and metabolism
E	120.0	8.28	Amino acid transport and metabolism
F	54.0	3.73	Nucleotide transport and metabolism
H	96.0	6.63	Coenzyme transport and metabolism
I	63.0	4.35	Lipid transport and metabolism
P	70.0	4.83	Inorganic ion transport and metabolism
Q	26.0	1.79	Secondary metabolites biosynthesis, transport and catabolism
R	164.0	11.32	General function prediction only
S	89.0	6.14	Function unknown
-	1050	43.80	Not in COGs

assembly because chimeric reads will have no or low short sequence coverage. At the end of the process, only the best preassembled reads without artifacts are sent to the assembler [52].

After quality analysis and eliminating some of the preassembled reads by PBDAG-Con, the remaining 6,009 reads were fed into the Celera assembler which uses an overlap-layout-consensus strategy [49]. A total of 2 contigs were generated with sizes 1,647,861 bp and 1,076,634 bp. These contigs underwent an additional polishing step where they were compared against the raw reads and any artifacts found were removed [49]. The final consensus generated was analyzed and improved by using the multi-read consensus algorithm Quiver. Quiver takes the two contigs and the initial sequencing reads and maps the reads onto the assemblies [49]. It then disregards the alignment between the reads and the assemblies and a consensus is created independently from the reads allowing it to remove any fine-scale errors made by the Celera assembler [52]. An approximate copy of the consensus sequences is then generated by Quiver which makes insertions and deletions and those that improve the maximum likelihood are applied to the initial consensus sequence [53]. The two final contigs generated by Quiver were 1,648,613 bp and 1,077,094 bp.

The two contigs underwent a finishing process using SeqMan Pro (DNASTAR Inc., Madison, WI). The two contigs were collapsed into one and the sequence was then opened in a region homologous to the Ori of *F. psychrophilum* JIP02/86 resulting in another two contigs. These were resealed using SeqMan Pro to create one final complete contig.

Genome annotation

The NCBI Prokaryotic Genome Annotation Pipeline was used to predict protein coding genes, structural RNAs (5S, 16S, 23S), tRNAs, and small non-coding RNAs [54]. Protein coding genes were predicted by protein alignment using ProSplign [55] where only complete alignments with 100% identity to a reference protein are kept for final annotation. Frameshifted or partial alignments were further analyzed by GeneMarkS+ [56] for further analysis and gene prediction. A BLASTN search against a reference set of structural RNA genomes from the NCBI Reference Sequence Collection was conducted to find the structural RNAs since they are highly conserved in closely related prokaryotes. tRNAscan-SE was used to identify the tRNAs [57]. Small RNAs were predicted using a BLASTN search against sequences of selected Rfam families and the results were refined further using Cmsearch [58]. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) were identified by searching the CRISPR database with the CRISPRfinder program (http://crispr.u-psud.fr/Server/) [59-62].

Genome properties

The 2,715,909 bp (32.75% G+C) genome of *F. psychrophilum* ATCC 49418^T contains 6 rRNA operons and 49 tRNA genes and is predicted to encode 2329 proteins (Figure 3

Table 5 Some putative virulence factors of *F. psychrophilum* ATCC 49418

Locus tag	Gene name	Family	Product
FPG3_00455		M50	Putative zinc metalloprotease
FPG3_01260	*fpp1*	M12B	Psychrophilic metalloprotease Fpp1 precursor
FPG3_01265	*fpp2*	M43	Psychrophilic metalloprotease Fpp2 precursor
FPG3_06120		Zn Peptidase	Putative neutral zinc metallopeptidase
FPG3_06485	*hlyD*	HlyD2	Putative hemolysin D transporter
FPG3_10400	*hlyD*	HlyD2	Putative hemolysin D transmembrane transporter
FPG3_00420		MntH	Mn^{2+} and Fe^{2+} transporter of the NRAMP family
FPG3_00490		FeoA	Iron transport protein A
FPG3_00495		FeoB	Iron transport protein B
FPG3_04340		Peptidase M75	Iron-regulated protein A precursor
FPG3_04455		TM-ABC Iron Siderophore	ABC iron transporter system, permease component
FPG3_05120		FeoB	ABC iron transporter system, binding protein precursor
FPG3_06195		CCC1	Probable iron transporter
FPG3_09395		Plant peroxidase like	Hydroperoxidase with catalase and peroxidase activities
FPG3_00925		LRR5	Cell surface protein precursor with leucine rich repeats
FPG3_00930		LRR5	Cell surface protein precursor with leucine rich repeats
FPG3_00935		LRR5	Cell surface protein precursor with leucine rich repeats
FPG3_00940		LRR5	Cell surface protein precursor with leucine rich repeats

and Table 3). No plasmids were identified during the annotation process. The distribution of genes into COG functions is shown in Table 4. When compared to the JIP02/86 strain, ATCC 49418^T had fewer proteins classified as "not in COGs" (43.8% vs. 47.5%) and had slightly more replication, repair, and recombination COGs (96 vs. 82). The two strains differed little in other COG categories. The Average Nucleotide Identity (ANI) between ATCC 49418^T and JIP02/86 was calculated to be 99.34% (+/−1.83%) and 99.37% (+/− 1.71%) one way and 99.43% (+/− 1.51%) two way [63]. The estimated distance to distance hybridization (DDH) values between the two strains was calculated to be 96.20% (+/− 1.16%) and the distance was 0.0053. The probability that DDH>70% (i.e. same species) is 97.48% [64].

Insights into the genome sequence

A number of studies have been done to determine the pathogenesis of *F. psychrophilum* but, to date, the exact mechanisms are still unknown [1]. Some putative and previously characterized virulence factors are listed in Table 5. Proteolytic enzymes are widely used by fish pathogens to cause tissue damage and allow invasion of the host [1]. In the *F. psychrophilum* ATCC 49418^T genome there are four metalloprotease encoding genes including a predicted zinc metalloprotease [FPG3_00455], a predicted zinc peptidase [FPG3_06120] and the previously reported Fpp1 [66] and Fpp2 [67] metalloproteases. Rainbow trout with RTFS are anemic and past studies have reported that the red blood cells of rainbow trout are partially lysed when infected by *F. psychrophilum* [68,69]. Homologs of two RTX hemolysin transporters (FPG3_06485, FPG3_10400) were identified, but did not appear to be linked to any toxin or modification genes [70]. Six iron transport genes were also identified; these were anticipated since iron uptake is a well-known characteristic of most pathogens. Moreover, recent research has shown that attenuated *F. psychrophilum* strains cultured under iron limiting conditions confer greater protection to fish when used as an experimental vaccine [71]. A hydroperoxidase with predicted catalase and peroxidase functions were also identified. In addition, there are 11 cell surface proteins with leucine rich repeats that are predicted to be adhesins; several are listed in Table 5. These were very similar to the ones found in *F. psychrophilum* JIP/02. Further research is required to determine what functions these adhesins have and how they help *F. psychrophilum* bind to the host.

Conclusion

Flavobacterium psychrophilum, the causative agent of BCWD and RTFS in salmonid fishes, causes significant economic losses in the aquaculture industry. The genome sequence of the ATCC 49418^T strain will hopefully provide new insights into virulence mechanisms and pathogenesis of *F. psychrophilum* and help in the identification of suitable targets for vaccines and antimicrobial agents; however, to do this much more analysis will be required.

Abbreviations

BCWD: Bacterial cold water disease; RTFS: Rainbow trout fry mortality syndrome.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AW participated in genome sequencing analysis, bioinformatics analysis, drafted the original manuscript, and participated in the revision process. AK participated in genome sequence analysis and assembly refinement. JL and BD participated in the study design and provided funding for the project. JM conceived the study, provided funding for the project, and participated in the revision process. All authors read and approved the final manuscript.

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Author details

[1]Department of Pathobiology, Ontario Veterinary College, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada. [2]Department of Biology, 200 University Avenue West, Waterloo, Ontario N2L 3G1, Canada.

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