Title

In vivo experiments provide evidence that *Flavobacterium psychrophilum* strains belonging to multilocus sequence typing clonal complex ST191 are virulent to Rainbow Trout (*Oncorhynchus mykiss*)

Running title

Virulence of clonal complex-ST191 *Flavobacterium psychrophilum*

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Conflict of Interest

All authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD), causes significant economic losses worldwide, particularly in farmed Rainbow Trout *Oncorhynchus mykiss*. Over the last decade, multilocus sequence typing (MLST) has revealed >30 clonal complexes (CCs) globally, comprised of >320 *F. psychrophilum* sequence types (STs). Despite the large number of CCs worldwide, CC-ST10, which is currently the largest CC affecting Rainbow Trout, has been the primary focus of *F. psychrophilum* virulence studies, leaving the role of other CCs as primary causes of BCWD epizootics unclear. To this end, fingerling Rainbow Trout were experimentally challenged with *F. psychrophilum* strains belonging to the CC now recognized as the second largest in the world (e.g., CC-ST191) alongside CC-ST10 strains. Cumulative percent mortality was 100% in 7 month old Rainbow Trout and between 27.8% and 61.1% in 8 month old Rainbow Trout, respectively. All examined *F. psychrophilum* STs were virulent to Rainbow Trout and no significant differences in virulence between CC-ST10 and CC-ST191 were detected. Due to their wide distribution and high pathogenic potential, both CC-ST191 and CC-ST10 *F. psychrophilum* strains are excellent candidates for further research aimed at preventing and controlling BCWD.

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD; Borg 1948), generates heavy losses in farmed Rainbow Trout *Oncorhynchus mykiss*, worldwide (reviewed in Loch and Faisal 2017). Multilocus sequence typing has revealed substantial global *F. psychrophilum* genetic diversity, with at least 320 sequence types (STs) clustered in 31 clonal complexes (CCs; Nicolas et al. 2008; Fujiwara-Nagata et al. 2013; Nilsen et al. 2014; Knupp et al. 2019; Sebastião et al. 2020). Empirical observations from field studies suggest that isolates belonging to some CCs differ in host species preference (Nicolas et al. 2008; Van Vliet et al. 2016, Knupp et al. 2019), virulence (Nilsen et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019), serotype (Wakabayashi et al.1994; Rochat et al. 2017), and frequency of association with disease outbreaks (Nilsen et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019). As a prime example, CC-ST10 strains are the predominating cause of BCWD outbreaks, almost exclusively in farmed Rainbow Trout, worldwide (Nicolas et al. 2008; Avendaño-Herrera et al. 2014; Nilsen et al. 2014). Experimental infection studies with CC-ST10 strains confirmed these field observations, whereby exposed Rainbow Trout experienced high mortality and morbidity (Long et al. 2014; Sundell et al. 2019). Recently, our studies and those of others, reported the isolation of multiple strains from another MLST-CC that is now recognized as the second largest; CC-ST191, which have been isolated from diseased and apparently healthy farmed and wild-caught Rainbow Trout from across North America and Europe (Siekoula-Nguedia et al. 2012; Strepparava et al. 2013; Nilsen et al. 2014; Knupp et al. 2019). Contrary to CC-ST10 strains, however, laboratory-controlled experiments confirming the virulence of most CC-ST191 strains have not been performed. This is particularly important since this bacterium is frequently isolated alongside other fish pathogens (Evensen and Lorenzen 1997; Ma et al. 2019a), including

other flavobacterial species (Kämpfer et al. 2012; Loch et al. 2013; Loch and Faisal 2015), making its role as the primary etiological agent inconclusive.

To begin addressing this knowledge gap and better illuminate the epidemiology of BCWD-causing *F. psychrophilum* strains, we evaluated the *in vivo* virulence and disease course of four *F. psychrophilum* isolates belonging to the second largest and globally widespread CC (e.g., CC-ST191; Knupp et al. 2019) alongside three isolates belonging to the largest and most widespread CC, CC-ST10.

[A]Methods

[C] *Experimental challenges*. — Industry-standard, embryonated Rainbow Trout eggs were procured, hatched in flow-through systems, and reared to 7 months old (mo; median 18g) and 8mo (median 44g). Fish were fed *ad libitum* daily and supplied dechlorinated, degassed and oxygenated well-water (12ºC ± 1ºC) that is ultraviolet treated and passed through sand filters in aerated flow-through tanks; tanks were siphoned twice daily to remove uneaten food and detritus. Prior to experimental challenge, arbitrarily selected individuals were confirmed *F. psychrophilum* negative via culture.

In Experiment 1, 7mo Rainbow Trout were intramuscularly (IM) challenged with one of seven isolates belonging to one of five genotypes from either CC-ST10 or CC-ST191 (Table 1). Isolates were revived from cryostock on mTYES agar (Michel et al. 1999) and incubated at 15ºC for 72h; single colonies were then subcultured on fresh mTYES agar and incubated for 72h at 15ºC to verify purity. Colonies of each isolate were inoculated into mTYES broth and incubated at 15ºC with constant shaking at 100 rpm for 72h. Bacterial pellets were harvested via

centrifugation (2,571 x g, 15 min), re-suspended in sterile saline (0.65% NaCl), and adjusted to an optical density at 600-nm (OD_{600}) corresponding to ~1 x 10^9 CFU/mL^{-1} using a Biowave CO8000 Cell Density meter (WPA Inc., Cambridge, UK). To verify concentrations, bacterial suspensions were serially diluted ten-fold in 0.65% NaCl and plated in duplicate on mTYES agar, incubated at 15ºC for 7d, and final colony counts performed. Rainbow Trout (*n* = 5 fish/isolate/aquarium, in triplicate) were anesthetized in sodium bicarbonate-buffered tricaine methanesulphonate (MS-222; 100 mg L^{-1}) and IM injected at the base of the dorsal fin with a mean dose of 2.6 – 9.5 x 10^6 CFU/g/$fish^{-1}$ in a 50 µL volume of each respective isolate or 0.65% NaCl (negative control fish; *n* = 5 per aquarium, in triplicate). Fish were held in their respective flow-through tanks (12ºC ± 1ºC), cared for as described above, and monitored for 28d.

In Experiment 2, isolates representing two *F. psychrophilum* genotypes from CC-ST10 and CC-ST191 (Table 1) were utilized for IM challenges in 8mo Rainbow Trout. Isolates were prepared as described above, except OD_{600} was adjusted to 10^8 CFU/mL^{-1}. Rainbow Trout (*n* = 9 fish/isolate/aquarium, in duplicate) were anesthetized and IM injected as described above with a mean dose of 1.0 – 4.2 x 10^5 CFU/g/$fish^{-1}$, whereas negative control fish were IM injected with 0.65% NaCl (*n* = 9 fish per aquarium, in duplicate). Fish were held in their respective tanks (12ºC ± 1ºC) for 35d and cared for as described above.

In both experiments, dead and surviving fish were necropsied, clinically examined, and tissues from external lesions, gills, spleen, kidney, brain, heart, and liver were bacteriologically analyzed for *F. psychrophilum*; representative isolates from the treatments were confirmed as *F. psychrophilum* using endpoint PCR assay (Toyama et al. 1994) as described elsewhere (Van

Vliet et al. 2015). To confirm MLST genotype, two MLST loci that differentiate between the selected STs (e.g., *gyrB* and *tuf*) were PCR-amplified from representative recovered isolates and sanger-sequenced as previously described (Knupp et al. 2019). All challenge experiments were conducted in accordance with the MSU-Institutional Animal Care and Use Committee (AUF:201800132).

[C] *Data analysis*. — Cumulative percent mortality (CPM) and mean days to death (MDD) were calculated for each isolate and genotype in both experiments. Differences in CPM and MDD among isolates and genotypes were tested according to a completely randomized design with subsampling (experimental unit=aquaria; observational unit=individual fish). Fish mass was Z-score standardized and included as a continuous covariate because size can affect *F. psychrophilum* mortality (Madsen and Dalsgaard 1999). Differences in MDD between isolates were tested with the Tukey-Kramer method. Differences between STs were tested using linear contrasts of means. The Type-I error rate for testing was set at 0.05. Analyses were conducted in SAS® Version 9.4.

[A]Results and Discussion

Although immersion challenge is believed to be a more natural exposure route, intramuscular injection is highly reproducible and the preferred method for evaluating *F. psychrophilum* virulence in relatively larger Rainbow Trout (>5 - 10g; Garcia et al. 2000; Fredriksen et al. 2013). Using IM challenge, the seven *F. psychrophilum* isolates from CC-ST10 and CC-ST191 caused 100% mortality within seven days (e.g., ranging from two to seven days) in all Experiment 1 treatment groups. This fulminant mortality suggested that the representative

CC-ST191 and CC-ST10 isolates were highly virulent to Rainbow Trout, especially because the infectious dose (e.g., mean dose of 10^6 CFU/g/fish^{-1}) was consistent with other *F. psychrophilum* experimental challenges that led to mortality rates ranging from 25% to 97% (Madsen and Dalsgaard 1999; Garcia et al. 2000; Fredriksen et al. 2013; Ma et al. 2019b).

In Experiment 2, where the infectious dose was one log lower (i.e., mean dose of 10^5 CFU/g/fish^{-1}), mean CPM ranged from 27.8% to 61.1% and 38.9% to 50% for CC-ST191 and CC-ST10 isolates, respectively. When isolates were grouped according to ST, mean CPM ranged from 33.4% to 50.0% (Table 2). Likewise, MDD was similar among Experiment 2 isolates, where it ranged from 8.7 to 11.6 days and 9.5 to 10.8 days for CC-ST191 and CC-ST10 isolates, respectively (Table 2). No statistically significant differences in either CPM or MDD were detected among the evaluated isolates or STs (Table 2).

Similar to CC-ST10 isolates, gross pathological signs were apparent in fish challenged with all CC-ST191 strains, suggesting that CC-ST191 is pathogenic to Rainbow Trout. Indeed, every dead fish in both experiments exhibited characteristic signs of BCWD, including multifocal to diffuse or focally extensive skin ulcerations that progressed into the underlying muscle and were often accompanied by a yellowish discoloration (Figure 1A,B). Additional disease signs included petechia and ecchymoses within the eye, isthmus, fins, ovaries, swim bladder, heart, and liver, and severe gill pallor with concurrent diffuse petechia. A yellow pigmented bacterium morphologically consistent with *F. psychrophilum* was recovered from all cultured external lesions and internal organs (e.g., brain, heart, kidney, liver, and spleen), and representative isolates from each treatment group were confirmed as *F. psychrophilum* via endpoint PCR (Van Vliet et al. 2015) and as the original ST via sequence analyses. No

mortalities occurred in the negative control groups, nor were any yellow pigmented bacteria recovered.

Despite CC-ST191 having been linked to disease outbreaks across the USA (Knupp et al. 2019) and in at least five European countries (Siekoula-Nguedia et al. 2012; Strepparava et al. 2013; Nilsen et al. 2014), little is known about the virulence and disease course of this multi-continental CC under controlled environmental conditions. In fact, only one recent study has incorporated a CC-ST191 isolate for virulence assessment. In the study by Ma et al. 2019b, Rainbow Trout (3.5g) challenged with CC-ST191 isolate US54 led to ≈62% mortality. The sum of our results provide evidence that multiple isolates belonging to the two largest STs within CC-ST191 appear equally virulent to larger (i.e., 18g and 44g) Rainbow Trout (Table 1) as two predominant STs within CC-ST10, which is by far the largest and most geographically widespread and reported MLST CC not only within the USA (Van Vliet et al. 2016; Knupp et al. 2019; Sebastião et al. 2020), but also globally (Siekoula-Nguedia et al. 2012; Strepparava et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014). Studying the phenotypes, including the virulence of CC-ST191 *F. psychrophilum* isolates, is imperative for developing widely applicable, efficacious BCWD prevention and control strategies. However, many *F. psychrophilum* phenotypic studies have focused solely on CC-ST10 isolates, which have proven to be quite heterogenous. In this context, *F. psychrophilum* from CC-ST10 have been found serologically (Rochat et al. 2017) and genetically (Duchaud et al. 2018) diverse, with variability in *in vitro* antibiotic susceptibility (Van Vliet et al. 2017) and proteolytic enzyme profiles (Rochat et al. 2019). Whether *F. psychrophilum* from the second largest MLST CC (i.e., CC-ST191) are also heterogenous in similar aspects remains to be determined.

In summary, this study has expanded our understanding of CC-ST191, the second largest *F. psychroph*ilum MLST CC affecting Rainbow Trout worldwide. This effort demonstrated the examined strains are just as virulent as those in CC-ST10 and builds upon previous work, augmenting the current knowledge of *F. psychrophilum*. These results provide critical foundational information for the study of other globally relevant MLST CCs, the study of which will be crucial for developing BCWD prevention and control strategies for predominating *F. psychrophilum* strains.

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Table legends

Table 1. Metadata and multilocus sequence typing-genotype for the seven *Flavobacterium psychrophilum* isolates used in this study. All *F. psychrophilum* isolates were originally recovered from Rainbow or steelhead Trout *Oncorhynchus mykiss*.

Table 2. Results of *Flavobacterium psychrophilum* Experiment two. Six *F. psychrophilum* isolates from four distinct multilocus sequence typing genotypes were utilized. The results of the challenge are listed first and include cumulative percent mortality with standard error (SE), and mean days to death with SE. Following the first section of the table are the results of the challenge by genotype (i.e., isolates belonging to same genotype were grouped for analysis). No significant differences in cumulative percent mortality or mean days to death were appreciated.

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Isolate	U.S. state of isolation	Location of isolation	Year of isolation	Clonal complex (cc)	Sequence type (st)	Reference
US75[a,b]	Pennsylvania	SFH2	2016	CC-ST10	ST10	Knupp et al., 2019
US26[a,b]	Michigan	SFH4	2010	CC-ST10	ST78	Van Vliet et al., 2016
US87[a]	Michigan	SFH4	2016	CC-ST10	ST275	Knupp et al., 2019
US215[a,b]	Michigan	WE1	2017	CC-ST191	ST267	Knupp et al., 2019
US54[a,b]	Michigan	SFH4	2013	CC-ST191	ST267	Van Vliet et al., 2016
US181[a,b]	Pennsylvania	SFH1	2015	CC-ST191	ST301	Knupp et al., 2019
US343[a,b]	West Virginia	PF2	2014	CC-ST191	ST301	Knupp et al., 2019

[a] Isolate used in experiment 1

[b] Isolate used in experiment 2

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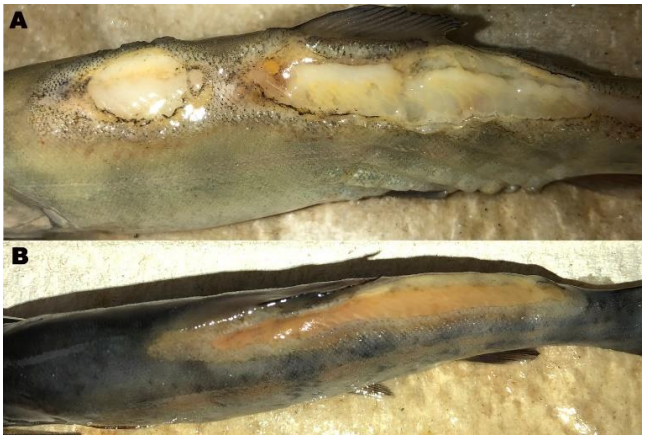
Clonal complex (cc)	Sequence type (st)	Isolate	Cumulative percent mortality ± se	Mean days to death ± se
		Sham-inoculated	00.0 ± 0.00	N/A
CC-ST10	ST10	US75	38.9 ± 5.57	09.5 ± 0.80
	ST78	US26	50.0 ± 5.57	10.8 ± 0.07
CC-ST191	ST267	US215	61.1 ± 5.57	11.6 ± 0.77
		US54	33.3 ± 0.00	08.7 ± 0.07
	ST301	US181	27.8 ± 5.57	10.5 ± 0.13
		US343	38.9 ± 5.57	10.6 ± 0.53
CC-ST10	ST10	US75	38.9 ± 5.57	09.5 ± 0.80
	ST78	US26	50.0 ± 5.57	10.8 ± 0.07
CC-ST191	ST267	US215, US54	47.2 ± 2.79	10.2 ± 0.42
	ST301	US181, US343	33.4 ± 5.57	10.6 ± 0.33

Figure legend

Figure 1. Gross disease signs in Rainbow Trout *Oncorhynchus mykiss* experimentally challenged with *Flavobacterium psychroph*ilum strains from multilocus sequence typing clonal complexes (CC) ST10 (A) or ST191 (B): A) Severe multifocal to diffuse ulceration and B) Severe focally extensive ulceration of the dorsum, both of which penetrate into the underlying muscle and are accompanied by a grossly appreciable yellowish discoloration.



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