Title

Host specific preference of some *Flavobacterium psychrophilum* multi-locus sequence typing genotypes determines their ability to cause bacterial coldwater disease in coho salmon (*Oncorhynchus kisutch*)

Running title

F. psychrophilum MLST variant host preference

Authors

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Acknowledgements

This work was supported in part by USDA Agriculture and Food Research Initiative competitive grant no. 2016-67015-24891. We also appreciate the financial support provided by Michigan State University AgBioResearch to TPL through the Stanislaus F. Snieszko Endowed Scholar program.

Conflict of Interest

All authors declare that they have 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 causes bacterial coldwater disease (BCWD) in salmonids, resulting in significant losses worldwide. Several serotyping and genetic studies of *F. psychrophilum* have suggested some geno-/serotypes may be either host specific or generalistic in nature; however, this association has not been adequately explored *in vivo* using more natural exposure routes. Herein, *F. psychrophilum* isolate US19-COS, originally recovered from coho salmon (*Oncorhynchus kisutch*) and belonging to multi-locus sequence typing clonal complex (CC) CC-ST9, and isolate US53-RBT, recovered from rainbow trout (*O. mykiss*) and belonging to CC-ST10, were serotyped via PCR, evaluated for proteolytic activity, and utilized to determine their median lethal dose in immersion-challenged coho salmon fingerlings. US19-COS belonged to serotype 0, hydrolyzed casein and gelatin but not elastin, led to fulminant multiorgan infections, and elicited severe gross and microscopic pathology. In contrast, US53-RBT, belonging to serotype 2, hydrolyzed all three substrates, but did not lead to detectable infections, disease signs, or mortality in any exposed coho salmon despite proving virulent to rainbow trout in previous experiments. This study provides *in vivo* evidence for potential host specificity of some *F. psychrophilum* genotypes that can also be serologically distinct, a matter of importance towards better understanding *F. psychrophilum* disease ecology and epidemiology.

1. Introduction

Flavobacterium psychrophilum, causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome, causes substantial economic losses in salmonid aquaculture facilities and hatcheries worldwide (reviewed in Loch & Faisal, 2017). Although all salmonid species (Family *Salmonidae*) are considered susceptible, coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*O. mykiss*) are particularly vulnerable to BCWD epizootics (Holt, 1987). Indeed, *F. psychrophilum* was first isolated from a disease outbreak resulting in mass mortality of juvenile coho salmon in the Pacific Northwest region of the United States in the 1940s (Borg, 1948); similar disease outbreaks affecting rainbow trout in the eastern USA were described by Davis (1946).

Since its initial isolation, multiple studies utilized a variety of methodologies to investigate the intraspecific diversity of *F. psychrophilum* for epidemiological purposes. Pacha (1968) and Holt (1987) used serological approaches to compare *F. psychrophilum* isolates recovered from different fish host species (e.g., coho salmon, Chinook salmon; *O. tshawytscha*, and brook trout; *Salvelinus fontinalis*) and locations across the USA (e.g., Oregon, New Hampshire, Michigan, and Alaska); their studies revealed both shared and distinct bacterial antigens, some of which varied by host species and location (Pacha, 1968; Holt, 1987). Subsequent serological studies of isolates recovered from multiple fish species on several continents led to descriptions of three to seven *F. psychrophilum* serotypes (Wakabayashi, Toyama, & Iidia, 1994; Lorenzen & Olesen, 1997; Izumi & Wakabayashi, 1999; Mata, Skarmeta, & Santos, 2002; Izumi, Aranishi, & Wakabayashi, 2003), some of which appeared to be associated with particular host species (Wakabayashi, Toyama, & Iidia, 1994; Izumi & Wakabayashi, 1999; Mata, Skarmeta, & Santos, 2002). More recently, Rochat et al. (2017) devised a reproducible multiplex PCR-based serotyping assay that revealed at least three molecular serotypes (Types 1-3) that overlap with the conventional serotypes described by

Lorenzen & Olesen (1997). In addition, Rochat et al. (2017) described a molecular "Type 0" comprised of a diversity of isolates that were genomically distinct from Types 1-3 because of their less conserved genomic structure. Since then, "Type-4" has been described, following further genomic analyses, by Avendaño-Herrera et al. (2020). Importantly, use of this scheme provided further evidence that some *F. psychrophilum* molecular serotypes appeared to be linked to infections in particular host species (i.e., Type-0 isolates are generally recovered from coho salmon, Type-1 isolates are typically recovered from rainbow trout, Type-3 isolates are recovered from ayu, *Plecoglossus altivelis*, and Type-2 and Type-4 are recovered from multiple species; Rochat et al., 2017; Sundell et al., 2019; Avendaño-Herrera et al., 2020).

Multilocus sequence typing (MLST) has also been used to investigate the epidemiology of *F. psychrophilum* after a scheme was devised by Nicolas et al. (2008). Therein and in subsequent studies, findings based upon observations from natural disease events have suggested that some *F. psychrophilum* MLST clonal complexes (CCs) preferentially infect certain host species (Nicolas et al., 2008; Fujiwara-Nagata et al., 2013; Avendaño-Herrera et al., 2014; Nilsen et al., 2014; Van Vliet et al., 2016; Knupp et al., 2019). Indeed, the largest and most widespread CC worldwide, CC-ST10, has been recovered almost exclusively from rainbow trout (Nilsen et al., 2014; Van Vliet et al., 2016; Knupp et al., 2019), whereas almost all *F. psychrophilum* isolates belonging to CC-ST9 have been recovered from coho salmon (Fujiwara-Nagata et al., 2013; Avendaño-Herrera et al., 2014; Van Vliet et al., 2016; Knupp et al., 2019).

Despite field-based evidence supporting the host specificity of some *F. psychrophilum* sero- and geno-variants, *in vivo* experiments testing this epidemiologically relevant hypothesis under controlled laboratory conditions have, for the most part, been only indirectly tested. By elucidating host-species preferences that may be dictated by *F. psychrophilum* serotype/genotype, it is possible that targeted and more efficacious BCWD control and

prevention (e.g., vaccination) methods can be devised. Herein, we report the molecular serotype, proteolytic activity, and virulence of two genetically distinct *F. psychrophilum* isolates from two globally relevant MLST CCs that putatively infect either coho salmon or rainbow trout.

2. Materials and Methods

2.1 *Flavobacterium psychrophilum* Isolate Selection

Two *F. psychrophilum* isolates recovered from systemically infected fish (e.g., US19 and US53) belonging to two MLST sequence types (STs; e.g., ST13 and ST78; Van Vliet et al., 2016) within two MLST CCs (e.g., CC-ST9 and CC-ST10) that have been detected on four continents (Knupp et al., 2019) were selected for this study. In addition to their global significance, both CCs have been recovered almost exclusively from one of two salmonid species: CC-ST9 from coho salmon (COS), and CC-ST10 from rainbow trout (RBT).

2.2 Molecular Serotyping

Because *F. psychrophilum* serotype has also been implicated in strain-host species preference, the molecular serotypes for *F. psychrophilum* US19-COS and US53-RBT were determined using the multiplex PCR (mPCR)-based serotyping scheme developed by Rochat et al. (2017) with minor modification to the reaction mixture. Briefly, each 50 µl mPCR reaction comprised 25 µl of 2X GoTaq® Green Master Mix (Promega), 20 ng of DNA template, 0.1 µM of each control primer, 0.5 µM of each primer used to identify each molecular serotype, with nuclease-free water composing the remainder. Sterile nuclease-free water served as a negative control, whereas *F. psychrophilum* type strain ATCC 49418^{T}, FP900406, and CSF259-93 served as positive controls for serotypes 0, 1, and 2, respectively. The mPCR cycling parameters of Rochat et al. (2017) were utilized in an Eppendorf® Mastercycler® pro thermal cycler. Five µl

of amplified PCR product was separated by electrophoresis in a 1.5% agarose gel prepared with 1X SYBR Safe DNA gel stain for 35 minutes at 100V, with 1-Kb Plus DNA Ladder (Thermo Fisher Scientific) as the molecular size standard. The gel was then viewed under UV transillumination to estimate amplicon size and assign mPCR serotype (e.g., Type-0, 188 bp; Type-1, 188 and 549 bp; Type-2, 188 and 841 bp; Type-3, 188 and 361 bp; Type-4, 188 and 992 bp; Rochat et al., 2017; Avendaño-Herrera et al., 2020).

2.3 Characterization of Proteolytic Activity

F. psychrophilum exhibits an array of proteolytic activities (Pacha, 1968), and some studies have suggested such proteases (e.g., caseinase, gelatinase, and elastase) may contribute to virulence (Bertolini et al., 1994, Madsen & Dalsgaard, 1999; Rochat et al., 2019), thereby potentially playing a role in host-species specificity. Thus, the proteolytic activities of US19-COS and US53-RBT were assessed alongside reference isolates ATCC 49418^{T} and CSF259-93 on tryptone yeast extract salts medium (TYES; Holt, 1987) agar supplemented with casein, elastin, or gelatin as previously described (Sundell et al., 2019). Briefly, isolates were revived from cryostock on TYES, which was modified according to Michel, Antonio, & Hedrick (1999) and is referred to hereafter as mTYES, and then incubated at 15ºC for 72h, after which cultures were visually inspected for purity. A 1-µl loopful of each isolate was inoculated into 1L of mTYES broth and incubated at 15ºC with constant shaking at 150rpm for 48h to achieve bacteria in a logarithmic phase of growth. Bacteria were harvested from mTYES broth via centrifugation (2,571 x g, 10min), rinsed in a sterile 0.65% saline suspension, 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 quantify flavobacterial concentrations, serial dilutions in ten-fold increments were plated on mTYES agar in duplicate and incubated at 15ºC for 7d, after

which final colony counts were performed. For each of the three proteolytic assays, 10 µl of bacterial suspension was spotted in triplicate onto the agar surface, allowed to dry, and then incubated for 7d at 15ºC. After incubation, the clear zone diameter, which is a summation of the colony diameter and the hydrolyzed portion of the medium, was divided by the colony diameter to produce a clear-zone ratio (CZR; Sundell et al., 2019).

2.4 *In Vivo* Assessment of Virulence to Coho Salmon

2.4.1 Origin of Fish for Challenge Experiments

Embryonated coho salmon eggs that had been surface disinfected with iodophor at 50 ppm for 30 min immediately after artificial egg fertilization and again at 100 ppm for 10 min were obtained from the Platte River State Fish Hatchery (Michigan, USA). Upon arrival at the Michigan State University – University Research Containment Facility, eggs were again iodophor disinfected at 100 ppm (10 min) and maintained in a vertical incubator supplied with dechlorinated pathogen-free water (12ºC ± 1ºC; ~19 L/min) until hatching. Sac-fry were transferred into aerated flow-through tanks (40 L; 12ºC ± 1ºC); upon commencement of exogenous feeding, fry were continuously fed a commercial trout diet (Skretting, The Netherlands) of appropriately sized food (e.g., starter crumble – 1.5mm) via automatic feeder. After 8 weeks, fish were fed by hand twice daily until satiation and the water volume was increased to 400 L (12ºC ± 1ºC). Throughout rearing, tanks were cleaned and siphoned 1-2x daily to remove detritus and any uneaten food. Prior to challenge, a subset of fish to be used in challenge experiments were screened for the presence of bacteria, including *F. psychrophilum* via culture, and verified to be free from infection.

2.4.2 *F. psychrophilum* Inoculum Preparation for Experimental Challenges

F. psychrophilum isolates US53-RBT and US19-COS were revived from cryostock on mTYES agar, incubated at 15ºC for 72h, and then visually inspected for purity. Each isolate was inoculated into 5L of mTYES broth, incubated, harvested, and adjusted to ~1 x 10^{10} CFU/mL^{-1} as described in section 2.3. The bacterial suspension was serially diluted ten-fold to create four *F. psychrophilum* suspensions corresponding to 10^8 – 10^5 CFU/ml as subsequently verified via plate counts.

2.4.3 Median Lethal Dose Experiments

F. psychrophilum isolates US53-RBT and US19-COS were assessed for their ability to infect and cause disease in coho salmon (five-month old; mean weight 6.3g) using *in vivo* immersion challenge experiments. Three-hundred-ninety-six coho salmon were anesthetized in sodium bicarbonate-buffered tricaine methanesulfonate (MS-222; Syndel, USA) at a concentration of 100mg L^{-1}, adipose fin-clipped using sharp sterile scissors (Holt, 1987), and then allowed to recover in aerated water. Fish (n = 22 per dose in duplicate) were subsequently immersed for 30 minutes in aerated water containing 10^8, 10^7, 10^6, or 10^5 CFU/ml of either US19-COS or US53-RBT, whereas control fish (n = 22 in duplicate) were immersed in an identical volume of water only. Following immersion challenge, fish were transferred into aerated flow-through glass tanks (37.85 L; n = 22 fish per tank in duplicate) supplied with dechlorinated pathogen-free water (12ºC ± 1ºC).

Fish were monitored daily for 34d and cared for as described previously; mortalities were necropsied, clinically examined, and multiple tissues (e.g., external ulcerations, gill, brain, heart, kidney, liver, and spleen) were bacteriologically analyzed for *F. psychrophilum* on mTYES agar. Terminally moribund and surviving fish (i.e., survived 34d post-challenge) were euthanized via

MS-222 overdose (250mg L^{-1}) and analyzed similarly. To estimate the median lethal dose (LD_{50}) for US19-COS and US53-RBT, the Reed-Muench method (Reed & Muench, 1938) was utilized. All challenge experiments were conducted in accordance with the MSU-Institutional Animal Care and Use Committee (AUF:201800132).

To confirm the identity of representative isolates from each challenge dose, the *F. psychrophilum* specific endpoint PCR assay of Toyama, Kita-Tsukamoto, & Wakabayashi, (1994) was utilized as described previously (Van Vliet, Loch, & Faisal, 2015). Similarly, to confirm the CC/ST of representative recovered *F. psychrophilum* isolates, two - three MLST loci that can differentiate ST13 (e.g., *trpB* and *tuf*) and ST78 (e.g., *gyrB*, *fumC*, and *tuf*) from other STs were PCR-amplified and sanger-sequenced as previously described (Knupp et al., 2019).

2.4.4 Histopathological Assessment

To begin to explore if *F. psychrophilum* strains US19-COS and US53-RBT vary in the tissue changes they elicit at the microscopic level, one challenged coho salmon from each treatment replicate was euthanized via MS-222 overdose at seven regular time points throughout the experiment, and fixed whole (after body cavity was opened using sterile scissors) in phosphate-buffered 10% formalin for 24h. Given the progression of morbidity and mortality (see section 3.3 below), however, only fish receiving the highest challenge dose were further processed for histopathological assessment, which entailed paraffin-embedding, microtome sectioning (5 µm), and staining with haematoxylin and eosin (H&E; Prophet, Mills, & Arrington, 1992). Slides were then examined via light microscopy.

2.5 Data Analysis

A one-way analysis of variance (ANOVA) with a Welch correction (to account for unequal within-group variances) was used to test mean CZR among the isolates for the evaluated media (i.e., caseinase, gelatinase, or elastase) as a measure of differences in proteolytic activity for the CZR. If the null hypothesis of no difference in mean CZR among the isolates was rejected, pairwise comparisons of mean CZRs between the isolates were conducted using pairwise two-sample, two-tailed *t*-tests assuming unequal variances with the Bonferroni correction for multiple comparisons ($\alpha = 0.05$). The ANOVA test and follow-up *t*-tests were performed in SAS® Version 9.4 using PROC GLM and PROC TTEST.

Differences in cumulative mortality among the isolates and dosages were tested with a generalized linear model assuming a beta family distribution and logit link function. Isolate and dosage were treated as factor variables and an isolate × dosage interaction term was included in the model. Each tank housing experimentally challenged fish was treated as the experimental unit in the model. Because the beta distribution assumes data to be > 0 and <1, tanks where no mortality occurred were assigned a cumulative mortality rate of 3.125% so that analyses could proceed. Because the isolate × dosage interaction term was found to be significantly different from 0 (see result below), the experimental data suggested that differences among the isolates depended on the dosage level. Consequently, we examined the simple effect differences between the isolates at each of the dosage levels. The generalized linear model was fit in SAS® Version 9.4 using PROC GLIMMIX.

3. Results

3.1 Molecular Serotype

Using the multiplex PCR assay developed by Rochat et al. (2017), *F. psychrophilum* isolate US19-COS yielded a 188bp amplicon and thus was assigned to molecular serotype Type-

0, whereas US53-RBT yielded two amplicons of 841bp and 188bp, corresponding to Type-2 (Figure 1).

3.2 Proteolytic Activity of *F. psychrophilum* strains US19-COS and US53-RBT.

F. psychrophilum isolates US53-RBT and US19-COS both proteolyzed casein and gelatin (Table 1); however, only US53-RBT showed elastase activity (Table 1). When compared to two reference strains belonging to the same two MLST CCs, similar results were found. For example, ATCC 49418^{T} (CC-ST9) and CSF259-93 (CC-ST10) both showed caseinase and gelatinase activity but only CSF259-93 proteolyzed elastin (Table 1). The variance-weighted ANOVA tests indicated that there were overall significant differences among the isolates in mean CZR for caseinase (F=10.15; df=3, 3.813; P-value=0.0270) and elastase (F=139.82; df=1, 3.885, P-value=0.0003). However, the null hypothesis of no difference in mean CZR among the isolates for gelatinase could not be rejected (F=1.08; df=3, 3.868; P-value=0.4556). Even though the null hypothesis of no difference in mean CZR among the isolates for caseinase was rejected, the pairwise t-tests comparing mean CZR between isolates did not detect any significant differences at a Bonferroni-corrected alpha of 0.0083. For elastase, mean CZR produced by US53-RBT (e.g., 2.83 ± 0.03) was significantly greater than the other tested isolates (US53-RBT vs. CSF259-93: t=11.82; df=3.885; P-value=0.0003; US53-RBT vs. US19-COS: t=55.0; df=2; P-value=0.0003; US53-RBT vs. ATCC 49418: t=55.0; df=2; P-value=0.0003). Likewise, mean CZR for CSF259-93 was significantly greater for elastase than for US19-COS (t=30.77; df=2; P-value=0.0011) and ATCC 49418 (t=30.77; df=2; P-value=0.0011). A test of the differences in mean CZR between US19-COS and ATCC 49418 could not be performed because the value of all observations were the same (Table 1).

3.3 Virulence of *F. psychrophilum* strains US19-COS and US53-RBT to Coho Salmon

Coho salmon immersed in the highest concentration (e.g., 10^8 CFU/ml) of *F. psychrophilum* strain US19-COS developed classical gross external signs of BCWD as early as four days post-challenge in the form of a shallow focal dermal ulceration of the caudal peduncle (Figure 2A). As the disease progressed, multifocal ulcerations on the caudal peduncle formed and deepened (Figure 2B) and additional focally extensive ulcerations of the rostrum became apparent (Figure 2C). In the most severely affected coho salmon, caudal peduncle necrosis extended deep into the musculature, leaving the underlying spinal processes exposed (Figure 2D). Additional external signs of BCWD included diffuse ecchymoses and petechiae of the gills and intraocular focal ecchymosis (Figure 2E-F). Likewise, US19-COS infected coho salmon developed multiple internal lesions, including severe splenic swelling (Figure 3A), severe perisplenic hemorrhage (Figure 3B), multifocal hepatic ecchymoses (Figure 3C), and severe hemorrhage within the pyloric caeca and the surrounding adipose tissue (Figure 3D). In coho salmon exposed to the second highest US19-COS concentration (e.g., 10^7 CFU/ml), similar external signs of BCWD were observed, including ulcerations of the rostrum and caudal peduncle as well as gill hemorrhage. Internally, visceral organs (e.g., heart, liver, and kidney) appeared severely pale, whereas the spleen retained its normal color (e.g., dark red) but was severely swollen. No gross lesions were noted in coho salmon challenged with the two lowest bacterial concentrations (e.g., 10^6 and 10^5 CFU/ml).

In stark contrast to coho salmon challenged with US19-COS, coho salmon challenged with the two highest concentrations (e.g., 10^8 and 10^7 CFU/ml) of US53-RBT remained apparently healthy throughout the experiment, as was also the case for coho salmon exposed to the two lowest *F. psychrophilum* concentrations (e.g., 10^6 and 10^5 CFU/ml). Likewise, no gross lesions were observed in any mock-challenged (i.e., negative control) coho salmon.

The cumulative percent mortality in coho salmon immersed in four different concentrations of US19-COS corresponding to 2.44 x 10^8 – 2.22 x 10^5 CFU/ml ranged from 0-93.3% (Figure 4). Mortality began four to eight days post-infection and peaked between days 12 and 16 (Figure 4). By comparison, no coho salmon immersed in *F. psychrophilum* isolate US53-RBT at any of the four bacterial concentrations (e.g., 2.44 x 10^8 – 3.33 x 10^5 CFU/ml) died throughout the course of the 34d experiment (Figure 4). Similarly, no mock-challenged (i.e., negative control) fish died prior to euthanasia at 34d post-challenge.

The isolate × dosage interaction term in the generalized linear model fit to the cumulative mortality data was statistically different from 0 (F=203.83; df=3,8; P<0.0001). As a result, we used the SLICE option in PROC GLIMMIX to evaluate the simple effect differences between the isolates at each of the dosage levels. At the highest dose (e.g., 10^8 CFU/ml), US19-COS caused significantly higher mortality in coho salmon than US53-RBT (*F*=964.5, df=1,8, *P*<0.0001). Similarly, at the second highest dose (e.g., 10^7 CFU/ml), US19-COS caused significantly higher mortality in coho salmon than US53-RBT (*F*=39.36, df=1,8, *P*=0.0002). Based on the experimental results, the LD_{50} for US19-COS was estimated to be 6.62 x 10^7 CFU, whereas the LD_{50} for US53-RBT could not be estimated but is expected to greatly exceed 2.44 x 10^8 CFU (i.e., the highest challenge dose; Table 1).

3.4 Infection Status in Coho Salmon Challenged with *F. psychrophilum* US19-COS and US53-RBT

In all coho salmon that died after immersion exposure to *F. psychrophilum* US19-COS, the bacterium was recovered in a pure form at intensities ranging from 10^4 – 10^5 CFU g^{-1} from multiple external lesions (e.g., skin/muscle ulcerations and gills) using calibrated inoculating loops. Similarly, pure cultures of *F. psychrophilum* were recovered from multiple internal organs

(e.g., brain, spleen, and kidney), at high intensities ranging from $10^3 – 10^5$ CFU g^{-1} of tissue. *F. psychrophilum* was also recovered from the kidneys of all survivors (i.e., 34d post challenge) that had been challenged with US19-COS at the highest dose. *F. psychrophilum* was not recovered from any survivors that were challenged with the remaining three concentrations. Molecular analyses confirmed bacterial identity as *F. psychrophilum* in all cases and sequencing confirmed the recovered *F. psychrophilum* to be ST13 (data not shown).

In contrast to fish challenged with US19-COS, *F. psychrophilum* was not recovered from any coho salmon exposed to US53-RBT, including at the highest exposure concentration (e.g., 2.44 x 10^8 CFU/ml). Likewise, *F. psychrophilum* was not detected from any mock-challenged coho salmon.

3.5 Histopathology in Coho Salmon Challenged with *F. psychrophilum* US19-COS and US53-RBT

Fish that had been exposed to the highest dose of US53-RBT were euthanized on days 0 (i.e., immediately after challenge), 1, 2, and 3 and multiple cross sections, including the eyes, gills, brain, internal organs and bone and cartilage were examined microscopically. The only lesion observed was localized tissue loss with minimal secondary inflammation at the site of the removed adipose fin (Figure 5A) consistent with the experimentally induced trauma. There were no other microscopic lesions in any of the examined samples and bacterial colonization was not observed. Fish that had been exposed to the highest dose of US19-COS were euthanized on days 0, 1, 2, 3, and 11 and multiple cross sections, including the eyes, gills, brain, internal organs and bone and cartilage were examined microscopically. Lesions were similar to those observed in fish exposed to US53-RBT in fish that had been euthanized on days 0, 1 and 2. However, in fish euthanized at day 3, there was severe necrosis of the whole adipose fin at the caudal peduncle

and replacement with fibrosis (Figure 5B). By day 11, the two examined fish had complete loss of the adipose fin at the caudal peduncle and epidermal ulceration had extended laterally and the necrotic surface was covered by large numbers of bacteria (Figure 5C). The inflammation extended deep into the underlying muscle causing a severe extensive necrotizing myositis and muscle loss (Figure 5D).

4. Discussion

Despite evidence from field-based studies suggesting that some *F. psychrophilum* sero- and/or genotypes preferentially infect certain salmonid species and that others may be more generalistic in nature (Nicolas et al., 2008; Fujiwara-Nagata et al., 2013; Avendaño-Herrera et al., 2014; Van Vliet et al., 2016; Rochat et al., 2017; Sundell et al., 2019; Knupp et al., 2019), *in vivo* experiments directly or indirectly testing such hypotheses have yielded mixed results to date. For example, Holt (1987) challenged coho salmon, Chinook salmon, and rainbow trout with a *F. psychrophilum* isolate (e.g., SH3-81) recovered from coho salmon that was only recently determined as belonging to MLST CC-ST9 (i.e., the same clonal complex containing the current study isolate, US19-COS; Van Vliet et al., 2016), and found this strain to be virulent to all three species. Similarly, Ekman & Norrgren (2003) found a *F. psychrophilum* isolate recovered from Atlantic salmon (*Salmo salar*) was virulent to three different salmonid species (e.g., Atlantic salmon, rainbow trout, and sea trout; *S. trutta* L.); however, the sero-/MLST genotype of this strain was not reported. In contrast, Nagai & Nakai (2011) found that isolates recovered from and virulent to ayu (*Plecoglossus altivelis*) were avirulent to red spotted masou trout (*O. masou ishikawae*), and isolates (e.g., OH-224, ST54 and OH-0519, ST55; Fujiwara-Nagata et al., 2013) recovered from masou salmon (*O. masou*) or red spotted masou trout were virulent to red spotted masou trout but not ayu. Likewise, Fredriksen et al. (2016) found a *F.*

psychrophilum isolate recovered from rainbow trout was avirulent to Atlantic salmon despite causing fulminant mortality in rainbow trout in a previous experiment (Fredriksen et al., 2013), and that an isolate recovered from Atlantic salmon was highly virulent to Atlantic salmon but weakly virulent to rainbow trout. However and of importance, in each of these studies, fish were experimentally exposed to *F. psychrophilum* using a form of injection (e.g., subcutaneous, intraperitoneal, or intramuscular), a route that is known to bypass a multitude of immune defense mechanisms that have been shown to play a role in differential pathogen susceptibility and thus may confound host-pathogen interaction studies (Fast et al., 2001; Fuochi et al., 2017; Dash et al., 2018).

To more closely mimic a natural route of infection, this study utilized immersion challenge in combination with clipping of the adipose fin, a practice commonly utilized in salmonid hatcheries to delineate hatchery fishes from their wild counterparts (Auld, Noakes, & Banks, 2019). By allowing two genetically and serologically distinct *F. psychrophilum* strains to interact with the front-line immune defense mechanisms of immersion-exposed coho salmon, the subsequent host-pathogen interactions could be more rigorously explored. In doing so, an isolate recovered from coho salmon (e.g., US19-COS) belonging to an MLST CC (CC-ST9; Nicolas et al., 2008) recovered nearly exclusively from this same species on four continents (Nicolas et al., 2008; Fujiwara-Nagata et al., 2013; Avendaño-Herrera et al., 2014; Nilsen et al., 2014; Van Vliet et al., 2016; Knupp et al., 2019) proved highly virulent to immersion-challenged coho salmon fingerlings as evidenced by the generation of systemic and long-lasting (e.g., at least 34 days post-challenge) infections, severe clinical signs and gross and microscopic BCWD lesions, and nearly 100% cumulative mortality in treatment groups receiving the highest challenge dose. In stark contrast, isolate US53-RBT, which was recovered from rainbow trout and belongs to a MLST CC (CC-ST10; Nicolas et al., 2008) that almost exclusively affects rainbow trout on four

406 continents (Nicolas et al., 2008; Siekoula-Nguedia et al., 2012; Strepparava et al., 2013;
407 Avendaño-Herrera et al., 2014; Nilsen et al., 2014; Van Vliet et al., 2016; Knupp et al., 2019)
408 was avirulent to coho salmon under our experimental conditions; proving incapable of
409 establishing detectable infections and of causing tissue damage, or mortality despite being
410 administered at a high concentration. Moreover, this isolate had previously proven virulent to
411 experimentally challenged rainbow trout in a previous study (Knupp et al., submitted).
412 Collectively, these findings provide *in vivo* evidence for an important facet of the disease
413 ecology and epidemiology of this globally significant bacterial fish pathogen; namely, that some
414 *F. psychrophilum* geno-variants have differential capacities to infect and cause subsequent
415 disease in specific salmonid hosts.

416 In a similar context, the serotype of some *F. psychrophilum* strains, as determined using
417 the mPCR-based serotyping scheme of Rochat et al. (2017) and that was based upon the
418 serotypes Fd, Fp^T, and Th described by Lorenzen & Olesen (1997), may also be tied to host
419 species associations (Wakabayashi, Toyama, & Iidia, 1994; Izumi & Wakabayashi, 1999; Mata,
420 Skarmeta, & Santos, 2002). Currently, the majority of *F. psychrophilum* isolates that have been
421 serotyped using this assay have been recovered outside of North America, where they belong to
422 serotypes 0 – 4, some of which appear to be more closely associated with rainbow trout (e.g.,
423 Type-1), ayu (e.g., Type-3), coho salmon (e.g., Type-0), or multiple fish species (e.g., Type-2
424 and Type-4; Rochat et al., 2017; Saticioglu et al., 2018; Sundell et al., 2019; Avendaño-Herrera
425 et al., 2020). In agreement with these studies, isolates US19-COS, originally recovered from
426 coho salmon (Van Vliet et al., 2016), and US53-RBT, originally recovered from rainbow trout
427 (Van Vliet et al., 2016), were found to belong to Type-0 and Type-2, respectively. This
428 congruence with previous studies is noteworthy given the lack of recently published serotyping
429 data available for *F. psychrophilum* isolates recovered from the USA in combination with how

genetically distinct many US isolates are from strains recovered elsewhere in the world (Knupp et al., 2019). Whether such relationships hold true for other isolates recovered from North America remains to be determined and highlights the need for comprehensive serotyping studies of North American *F. psychrophilum* isolates.

Although thoroughly characterizing *F. psychrophilum* pathogenesis at the cellular level and examining the molecular host and/or pathogen mechanisms behind host species-associations were not primary goals of this study, initial histopathological and *in vitro* proteolytic evaluations were conducted. Interestingly, isolates US19-COS and US53-RBT both proteolyzed gelatin and casein, but only the latter degraded elastin, which is an extracellular matrix protein and component of connective tissue (Sage, 1982). These same results held true for two reference isolates belonging to the same CCs (e.g., CC-ST9 and CC-ST-10; Table 1), as well as for the majority of *F. psychrophilum* CC-ST10 isolates analyzed by Sundell et al. (2019). Moreover, Rochat et al. (2019) found that all but one of the *F. psychrophilum* CC-ST10 isolates they analyzed carry a novel elastase gene and degraded elastin *in vitro* (Rochat et al., 2019). Sundell et al. (2019), Rochat et al. (2019), and Soule et al. (2005) all concluded that elastase activity appears to be correlated with genetic lineage and may provide an evolutionary advantage for rainbow trout-associated isolates. In the current study, the elastinolytic US53-RBT isolate was incapable of causing disease in coho salmon, whereas the elastase negative US19-COS was highly virulent and led to severe pathological changes and death in the same fish species. Thus, it is possible that elastase plays a role in virulence for some *F. psychrophilum* strains affecting rainbow trout, but clearly other mechanisms appear sufficient for virulence for at least one CC-ST9 strain with a penchant for coho salmon. In this context, Barbier et al. (2020) found that the type IX secretion system, which secretes multiple potential virulence factors (e.g., peptidases, adhesins, and gliding motility proteins) was important for *F. psychrophilum* virulence.

Histological findings showed US19-COS was highly virulent to coho salmon, as evidenced by causing severe necrosis and extensive necrotizing myositis at the caudal peduncle. Such histological changes are consistent with what has been reported for BCWD in other salmonid species (e.g., Atlantic salmon and rainbow trout; Ostland et al., 2000; Nilsen et al., 2011). In contrast to US19-COS, US53-RBT challenged fish only had superficial necrosis of the adipose fin, which was most consistent with its removal as part of the experimental design.

Even though coho salmon and rainbow trout are both particularly susceptible to *F. psychrophilum* infections (Nematollahi et al., 2003) and despite the global importance of coho salmon farming (FAO, 2020), most *F. psychrophilum in vivo* challenge studies have focused on rainbow trout (Holt, 1987; Madsen & Dalsgaard, 1999; Decostere, Lammens, & Haesebrouck, 2000; Long et al., 2013; Long et al., 2014; Sundell et al., 2019) rather than coho salmon (Holt, 1987; Bertolini et al., 1994), leaving *in vivo* challenge models for this species relatively understudied. However, this study demonstrated the utility of an immersion-based challenge model for studying BCWD in coho salmon that will likely be of benefit for future *in vivo* studies focused on this species. Results herein also provide some important context for the *F. psychrophilum* type strain (NCMB 1947^{T}), which belongs to the same ST as US19-COS (i.e., ST13; reported in Nicolas et al., 2008) and has been deemed avirulent by multiple researchers who have utilized it in rainbow trout studies (Madsen & Dalsgaard, 2000; Jarau et al., 2018; Sundell et al., 2019). Considering this study's findings, however, it is possible that *F. psychrophilum* NCMB 1947^{T} is not "equipped" to cause disease in rainbow trout, but rather has a preference for coho salmon, a hypothesis that is also supported by the seminal work of Holt (1987), who reported 98% mortality in yearling coho salmon challenged with this strain.

In conclusion, this study has provided *in vivo* evidence that some *F. psychrophilum* geno-variants exhibit host species preferences that lead to differential capacities to cause disease. This,

combined with varying serotypes, has important implications not only for the disease ecology and epidemiology of this bacterium of global significance, but also must be accounted for in ongoing and future BCWD vaccine efforts if they are to be effective in multiple salmonid species. Further research on the mechanisms behind such host species preferences, as well as any host specificity or genericity of other *F. psychrophilum* geno-/sero-variants is warranted and will likely be necessary to guide the development of targeted BCWD control strategies so that future BCWD losses can be more effectively mitigated.

5. References

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Tables

Table 1. *Flavobacterium psychrophilum* isolates used in this study for *in vivo* challenge against juvenile coho salmon (*Oncorhynchus kisutch)* and/or for the assessment of proteolytic activity, which is presented as a ratio of the clear zone diameter to the colony diameter (in mm) ± standard deviation (SD). Clear zone ratios for a particular enzyme (e.g., caseinase, gelatinase, or elastase) containing an identical symbol (e.g., *, **, ***) are not significantly different ($\alpha > 0.05$) and a ratio of 1.00 ± 0.00 indicates no protease activity.

					Mean protease clear zone ratio (SD)		
Isolate ID	Host of origin	ST[†]	CC[‡]	LD_{50}[§] (CFU)	Caseinase	Gelatinase	Elastase
US19-COS	Coho salmon	ST13	CC-ST9	6.62×10^7	1.46 (0.07)*	1.97 (0.03)*	1.00 (0.00)*
ATCC 49418^T	Coho salmon	ST13	CC-ST9		1.39 (0.02)*	2.09 (0.11)*	1.00 (0.00)*
US53-RBT	Rainbow trout	ST78	CC-ST10	$>2.44 \times 10^8$	1.82 (0.08)*	2.11 (0.08)*	2.83 (0.03)***
CSF259-93	Rainbow trout	ST10	CC-ST10		1.58 (0.04)*	1.93 (0.09)*	2.22 (0.04)**

[†] Sequence type

[‡] Clonal complex

[§] Median lethal dose

Figure legends

Figure 1. Molecular serotyping of *Flavobacterium psychrophilum* using a multiplex PCR developed by Rochat et al. (2017). *F. psychrophilum* experimental challenge isolate US19, which was recovered from a coho salmon (*Oncorhynchus kisutch*) and challenge isolate US53, which was recovered from a rainbow trout (*O. mykiss*), were identified as Type-0 and Type-2, respectively. Lanes: (Ladder) 1-Kb Plus DNA Ladder; (1) US19; (2) US53; (3) ATCC 49418^{T}, positive control for Type-0; (4) FP900406, positive control for Type-1; (5) CSF259-93, positive control for Type-2; (6) negative control.

Figure 2. Gross external lesions in coho salmon (*Oncorhynchus kisutch*) following immersion challenge with *Flavobacterium psychrophilum* isolate US19-COS. A) Focal dermal ulceration of the caudal peduncle. B) Multifocal deep dermal ulceration of the caudal peduncle. C) Deep, focally extensive ulceration of the upper jaw. D) Deep, focally extensive ulceration of the caudal peduncle and myonecrosis, exposing the underlying vertebral column. E) Diffuse petechiae and ecchymoses of the gill. F) Intraocular focal ecchymoses.

Figure 3. Gross internal lesions in coho salmon (*Oncorhynchus kisutch*) following immersion challenge with *Flavobacterium psychrophilum* isolate US19-COS. A) Severe splenic swelling and diffuse erythema of the adipose tissue. B) Perisplenic hemorrhage. C) Multifocal ecchymoses of the liver. D) Hemorrhage of the pyloric caeca and surrounding adipose tissue.

Figure 4. Mean cumulative percent mortality of coho salmon (*Oncorhynchus kisutch*; mean weight 6.3g) over a period of 34 days following immersion challenge with *Flavobacterium psychrophilum* isolates US19-COS and US53-RBT, which belong to multilocus sequence typing

genotypes ST13 (in CC-ST9) and ST78 (in CC-ST10), respectively. Standard error bars are shown.

Figure 5. Microscopic lesions in coho salmon (*Oncorhynchus kisutch*) following immersion challenge with *Flavobacterium psychrophilum* isolate US19-COS and US53-RBT, which belong to multilocus sequence typing genotypes ST13 (in CC-ST9) and ST78 (in CC-ST10), respectively. A) Necrosis of the superficial portion of the adipose fin at the caudal peduncle three days post-infection with *F. psychrophilum* isolate US53-RBT. B) Severe necrosis of the whole adipose fin at the caudal peduncle and replacement with fibrosis three days post-infection with *F. psychrophilum* isolate US19-COS. C) Complete loss of the adipose fin at the caudal peduncle and extensive necrotizing myositis with bacterial colonization 11 days post-infection with *F. psychrophilum* isolate US19-COS. D) Severe ulceration and extensive necrotizing myositis with bacterial colonization of the caudal peduncle 11 days post-infection with *F. psychrophilum* isolate US19-COS.