

Characterization of Novel *Flavobacterium* spp. Involved in the Mortality of Coho Salmon (*Oncorhynchus kisutch*) in their Early Life Stages

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Abstract

Salmonid species in the Laurentian Great Lakes basin suffer from early mortalities that are often associated with low survival rates of swim-up fry. Coho salmon (*Oncorhynchus kisutch*) alevins exhibiting a spinning swimming behavior and convulsions were presented to the Aquatic Animal Health Laboratory of Michigan State University. Twelve yellow-pigmented bacterial isolates that were motile via gliding were recovered from the brains of dead and moribund fish and identified as *Flavobacterium* spp. (designated CS: 29-31, 34-38, 40-43). 16S rRNA sequence analysis indicated that 9 of the 12 sequences were grouped into a single clade (CS29, 31, 34-38, 40, 41) and were essentially identical over the 676 aligned bases used in the phylogenetic analysis. This clade is closely related to the eel-pathogenic *Flavobacterium johnsoniae*-like YO60 recovered from South Africa. The remaining three isolates, CS30, CS42, CS43 were closest to *Flavobacterium* sp. Wuba46 (100%), *Flavobacterium* sp. EP125 (100%), and *Flavobacterium* sp. WB 4.3-15 (99.9%), all of which were reported from European waters. Experimental challenge of five month old coho salmon fry via intra-peritoneal injection with the CS36 isolate resulted in morbidity and mortality rates of approximately 10% in the two highest infection doses. Clinical signs included tachybranchia, hemorrhages, and fin erosion. Stained tissue sections from dead and moribund fish showed degeneration of kidney tubules, edema in the renal interstitial tissues, heterophilic cellulitis and myodegenerative changes within the caudal peduncle, and a proteinaceous exudate in the coelomic cavity. Based on this study, flavobacterial infections with these isolates, which were detected for the first time in North America, can potentially cause losses in yolk sac and swim-up coho salmon fry.

Keywords: *Flavobacterium* spp.; Coho salmon; 16S rDNA; Fish disease

Introduction

Fish in their early life stages are particularly prone to infections, toxic chemical insults, and dietary deficiencies [1]. This is particularly true for salmonid species in the Laurentian Great Lakes basin that endure low survival rates in their first year [2,3]. A subset of these early mortalities has been collectively called early mortality syndrome (EMS), referring to yolk-sac and swim-up fry mortalities associated with an erratic, spinning, swimming behavior [4]. In Europe, an identical syndrome (designated M74) has been noticed in Atlantic salmon (*Salmosalar*) and brown trout (*Salmotrutta*) from the Baltic Sea [5]. While the exact etiology of EMS and M74 has not been fully elucidated, it is believed to result from severely depressed thiamine levels [6-8]. The mechanism(s) by which thiamine deficiency leads to EMS is currently unknown.

Members of the genus *Flavobacterium* (Family Flavobacteriaceae, Phylum Bacteroidetes) pose a serious threat to wild and propagated fish stocks worldwide [9]. *Flavobacterium* spp. have been retrieved from diverse ecological niches such as soil, sediment, freshwater and marine water ecosystems, microbial mats, sea ice, mosquito guts, and diseased fish [10]. While many flavobacteria are harmless, some are opportunistic or obligate pathogens that cause infections and disease in a wide variety of organisms, including invertebrates, fish, and humans [10]. In addition to the well-known fish pathogenic flavobacteria, such as *F. columnare*, *F. psychrophilum*, and *F. branchiophilum*, a number of novel fish-pathogenic flavobacteria have recently emerged [11,12]. Some flavobacteria (e.g., *F. psychrophilum*) have also been connected to mortalities of rainbow trout (*Oncorhynchus mykiss*) in their early life stages [13,14]. To this end, we report on the phenotypic, genotypic, and

phylogenetic characteristics of multiple *Flavobacterium* spp. isolated from diseased coho salmon yolk sac and swim up fry with signs similar to EMS.

Materials and Methods

Fish and sampling

Four-week old coho salmon in the late yolk sac to early swim up stages of development were collected from groups in the Platte River State Fish Hatchery (Beulah, MI) suffering mortality associated with erratic, spinning swimming. All moribund fish were isolated in a single raceway, where mortality reached 30% within one week. Originally, gametes were collected from feral coho salmon runs returning to spawn at the Platte River Weir (Beulah, Michigan, Lake Michigan watershed) in the fall of 2003. As a prophylaxis against EMS, all coho salmon yolk sac fry at the state hatchery were treated with 750 ppm thiamine hydrochloride as recommended elsewhere [15-18].

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Received November 03, 2011; **Accepted** December 12, 2011; **Published** December 21, 2011

Citation: Faisal M, TP Loch, M Fujimoto, SA Woodiga, AE Eissa, et al. (2011) Characterization of Novel *Flavobacterium* spp. Involved in the Mortality of Coho salmon (*Oncorhynchus kisutch*) in Their Early Life Stages. J Aquac Res Development S2:004. doi:10.4172/2155-9546.S2-005

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To further investigate this case, 60 moribund and 60 apparently healthy fish (from another raceway) were shipped alive to the Aquatic Animal Health Laboratory at Michigan State University, East Lansing, MI, USA. The fish were euthanized with an overdose of tricainemethanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA) and then were subjected to thorough gross examination, as well as virology, bacteriology, and parasitology analyses according to the American Fishery Society Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens [19] and the World Animal Health Organization (OIE) Aquatic Manual [20].

Bacteriological analysis

Samples were aseptically collected from brains and kidneys after first disinfecting the outside of the fish with 70% ethanol. Due to the extremely small fish size, sterile inoculating needles (27 gauge) were used to collect brain and kidney tissues. All materials and reagents were purchased from Remel Inc. (Lenexa, KS) unless otherwise specified. Tissue samples were inoculated into Hsu-Shotts [21] and trypticase soy broth and incubated at 22°C for 48 hrs. Any broth cultures displaying turbidity were then sub-cultured onto Hsu-Shotts and trypticase soy agars (TSA) and incubated for 24-48 hrs at 22°C. Tissue samples were concurrently inoculated into cytophaga broth and incubated at 15°C for 7 days, at which point any broth cultures with turbidity were sub-cultured onto Cytophaga agar. For long-term storage, pure bacterial stock cultures were kept at -80°C in the appropriate fluid medium supplemented with 20% glycerol.

Characterization of recovered isolates

From the bacterial growth obtained from the brains of moribund fry, 12 isolates representing all observed bacterial morphologies were selected for subsequent characterization. Single colonies from the representative isolates were streaked onto Hsu-Shotts agar, purity verified under a dissecting scope, and then identified based on their cultural and morphological characteristics. Additional biochemical testing was performed on three isolates (CS30, CS36, and CS37) that included cytochrome oxidase, catalase reaction (3% H₂O₂), acid and gas production from glucose, indole production, hydrogen sulfide production, citrate utilization, Triple Sugar Iron reaction, ONPG (*o*-nitrophenyl- β -D-galactopyranoside), lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, esculin hydrolysis, phenylalanine deaminase (BD Scientific, Franklin Lakes, NJ), urease, starch hydrolysis, growth on MacConkey agar, gelatinase, and caseinase and lipase in a Hsu-Shotts agar base. All tests were inoculated with 24 hr old cultures and read within seven days post-inoculation. Moreover, the presence of flexirubin was determined using the potassium hydroxide test [22], and Congo red adsorption, which tests the presence of extracellular galactosamine glycan [23], was also performed. The activities of 19 different enzymes were examined using the API-ZYM kit (BioMerieux, Inc., Durham, NC) following the manufacturer's protocol and were incubated at 22°C for 24 hours.

Antibiotic susceptibility testing was performed on the 12 isolates using standard agar disk diffusion susceptibility testing [24] with both Mueller-Hinton and Hsu-Shotts agars. The assayed antibiotics and their respective concentrations were as follows: polymyxin-B (300 iu), oxytetracycline (30 μ g), trimethoprim-sulfamethoxazole (25 μ g), erythromycin (15 μ g), ampicillin (10 μ g), novobiocin (30 μ g), azithromycin (15 μ g), florfenicol (30 μ g), and the vibriostatic agent O/129 (2,4-diamino, 6,7-di-isopropyl pteridine; 10 μ g).

PCR amplification of 16S rDNA genes for sequencing

Genomic DNA was extracted from the 12 bacterial isolates using the DNeasy blood and tissue kit (Qiagen Inc., Germantown, MD) following the manufacturer's protocol. 16S rRNA genes were amplified using the universal bacterial primers 27F (5' - AGA GTT TGA TCM TGG CTC AG - 3') and 1387R (5' - GGG CGG WGT GTA CAA GGC - 3'). Each PCR mixture of 100 μ l contained 4 μ l template DNA (20ng μ l⁻¹), 0.2 μ M of each primer, 0.25 mM of each deoxynucleoside triphosphate, and 2.5 U of *Taq*DNA polymerase (Invitrogen Corp., Carlsbad, CA) in a final concentration of 10mM Tris-HCl, 50mM KCl, and 1.5mM MgCl₂. PCR was performed under the following cycle conditions: an initial denaturation step at 94°C for 5 min and 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, and extension at 72°C for 110s. A final extension step at 72°C for 7 min was performed. The PCR product was purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The purified PCR products were sequenced at the Research Technology Support Facility at Michigan State University. Partial sequences of the rDNA genes were obtained using the 27F primer. The sequences have been deposited at National Institutes of Health GenBank under the following accession numbers: FJ479755 for CS29, FJ479756 for CS30, FJ479757 for CS31, FJ479758, for CS 34, FJ479759 for CS35, FJ479760 for CS36, FJ479761 for CS37, FJ479762 for CS38, FJ479763 for CS40, FJ479764 for CS41, FJ479765 for CS42, and FJ479766 for CS43.

Phylogenetic analysis

The phylogenetic relationships were analyzed using MEGA version 4.0 [25]. Nucleotide sequences of the 12 isolates were exported to and aligned in the Ribosomal Database Project (RDP) [26] together with 25 publicly available strains in RDP. The aligned sequences were retrieved in MEGA and the phylogenetic relationships were inferred by the Neighbor-Joining algorithm [27] with the Kimura-2-parameter model [28] based on 676 bases of 16S rRNA sequences. The robustness of the topology was assessed by performing 1,000 bootstrap replicates.

Experimental challenge

An experiment was performed to assess the pathogenicity of *Flavobacterium* sp. strain CS36 that represented the dominant isolate recovered from the brains of moribund fry. Three hundred three-month post-hatch coho salmon fry (5.4 cm, 1.8 g average length/weight) from a lot without any previous disease signs/mortality events were obtained from the Platte River State Fish Hatchery. Prior to the infection study, 10 random coho salmon were sampled to assess *Flavobacterium* spp. infection status. The fish were allowed to acclimate to the new laboratory conditions until regular feeding was resumed. Fish were kept in flow-through PVC tanks at a flow rate of 1.26 L/min with the water temperature kept at 10°C+1°C.

The *Flavobacterium* sp. strain CS36 was revived in Hsu-Shotts broth (22°C for 48 hrs), streaked onto solid Hsu-Shotts agar media, a single colony inoculated into a 500 ml flask of Hsu-Shotts broth, and incubated at 22°C for 72 hrs while being stirred at 60 rpms on Daigger stir plates (Daigger & Company, Vernon Hills, Illinois). Broth cultures were then centrifuged at 3500 rpm, rinsed in fresh Hsu-Shotts broth three times, pellets resuspended in 0.85% sterile saline, serially diluted onto solid media for colony counts, and then immediately used for experimental infections.

Two experiments were carried out simultaneously. In both experiments, fish were exposed to three successive handling events [12], which entailed netting the fish to be infected for 10 seconds and then

releasing them back into their tank, immediately prior to challenge with the CS36 strain. In the first experiment, six groups of 10 fish each were used for intraperitoneal (IP) injection at doses of 3.0×10^7 cfu/100 μL^{-1} to 3.0×10^2 cfu/100 μL^{-1} via serial tenfold dilutions in sterile 0.85% saline. Each fish received the respective dose in 100 μL of 0.85% saline using a 22-gauge needle (Becton Dickinson, Franklin Lakes, NJ). The needle was inserted approximately 3-5 mm dorsal to the anal fin and directed cranially into the intra-peritoneal space. An additional group of 10 fish was intraperitoneally injected with 0.85% saline only (100 μL fish⁻¹) and served as a negative control. In the second experiment, a group of 30 fish was immersed in 5L of 0.85% saline water containing 3×10^7 cfu mL⁻¹ of CS36 for 60 min. An additional group (10 fish) was mock-challenged in 5L of 0.85% sterile saline for 60 min and served as a negative control.

Clinical signs and mortality were recorded for 28 days post-challenge. Dead fish were processed immediately for re-isolation and histopathology. At the end of the 28 day observation period, the experiment was terminated, with half of the surviving fish preserved whole in 10% buffered formalin for histopathological examination, and the remainder for clinical examination and bacterial re-isolation on both TSA and Hsu-Shotts agar.

Histopathological analyses

The microscopic tissue alterations in experimentally infected fish were assessed in hematoxylin and eosin stained tissue sections. Fish were preserved in 10% buffered formalin, embedded within paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin according to the methods described in [29]. In total, 8 negative control fish (4 injected with sterile saline and 4 immersed in sterile saline) and 11 infected fish (4 I.P. injected with 3.0×10^7 cfu 100 μL^{-1} , 3 I.P. injected with 3.0×10^6 cfu 100 μL^{-1} , and 4 immersion infected in 3.0×10^7 cfu mL⁻¹) were examined for histopathological alterations.

Results

Bacterial isolation and isolate characterization

Affected fish exhibited a spinning swimming behavior with convulsions and congregated around the water outlet. Within two weeks, percent cumulative mortality amounted to 30% in the affected raceway. Following 48 hours of incubation at 22°C, bacterial growth was noticed from brain and/or kidney samples of the almost every moribund or dead fish tested. Similarly, after 48 hours of incubation at 15°C in cytophaga broth, almost every culture was turbid. When sub-cultured onto Hsu-Shotts, trypticase soy, and cytophaga agars, bacterial growth was pure and profuse, yielding yellow colonies with irregular margins. Representative isolates from all 3 media types were Gram negative, gliding, filamentous rods with pointed ends (3.5 μm x 0.5 μm), that produced catalase, tested positive for flexirubin, were Congo red negative, and did not produce cytochrome oxidase. Based on these characteristics, the 12 bacterial isolates were tentatively identified as *Flavobacterium* spp. (designated CS29-31, 34-38, 40-43). No bacteria were recovered from the brains or internal organs of apparently healthy fish.

The phylogenetic affiliation of the 12 isolates was determined by comparative 16S rRNA sequence analysis. All 12 sequences were grouped within the genus *Flavobacterium* (Figure 1). Nine out of 12 sequences were grouped into a single clade (CS29, 31, 34-38, 40, 41) and were essentially identical over the 676 aligned bases used in the phylogenetic analysis (Figure 1a). This clade shared a 99.4% similarity with the previously described YO60 flavobacterial strain [12]. Sequence

similarity between this clade with another known *Flavobacterium* species, *F. pectinovorum*, was 98.4%. The remaining three isolates, CS30, CS42, CS43 were closest to the other previously described *Flavobacterium* spp. strains; Wuba46 (100%), EP125 (100%), and WB 4.3-15 (99.9%; Figure 1b).

Further phenotypic characterization was performed on the three isolates: CS30, CS36, and CS37 (Table 1). *Flavobacterium* spp. CS36 and CS37 demonstrated 100% similarity with one another. Strain CS30 differed from strains CS36 and CS37 in its ability to produce phenylalanine deaminase and lipase and its inability to utilize citrate. According to the API ZYM assay, CS30 was also dissimilar in that it produced a weak positive reaction for trypsin, and positive reactions for α -galactosidase and N-acetyl- β -glucosaminidase, but a negative reaction for β -galactosidase. However, by conventional tube tests, all three isolates were positive for β -galactosidase (Table 2). Phenotypic comparisons of *Flavobacterium* YO60 [12] with CS 36 and CS 37 showed that all exhibited gliding motility and produced caseinase, but differed in their ability to hydrolyze gelatin. No other phenotypic characteristics were reported by Flemming and colleagues for the strain YO60. Moreover, phenotypic characteristics for *Flavobacterium* sp. Wuba46 are not available and thus could not be compared to CS30. Phenotypic discrepancies between CS30 and *F. hydatis* included the former's inability to utilize glucose and the production of phenylalanine deaminase. The isolates were resistant to polymyxin-B, the vibriostatic agent O/129, ampicillin, and azithromycin, but were sensitive to oxytetracycline (20-23 mm), trimethoprim-sulfamethoxazole (15-24mm), erythromycin (12-19 mm), novobiocin (23-25 mm), florfenicol (25-29 mm).

Experimental infection

No *Flavobacterium* spp. or other bacterial pathogens, viruses, or parasites were detected in the 10 randomly sampled coho salmon prior to experimental challenge. In coho salmon I.P. injected with CS36, both morbidity and mortality were observed. Morbidity varied amongst groups, with the most severe signs, such as melanosis, tachybranchia, lethargy, erythema in the caudal fin (Figure 2), and caudal fin erosion and necrosis (Figure 3), occurring in fish groups intraperitoneally challenged with 3.0×10^7 cfu and 3.0×10^6 cfu (the two highest challenge doses). Mortalities peaked at 10% on day 5-post infection for the 3.0×10^7 cfu group and at 10% on day 8 for the 3.0×10^6 groups, respectively; no mortalities occurred in the remaining I.P. -infected groups. Gross pathology in mortalities included mild bilateral exophthalmia, ascites, severe hepatic and renal pallor, mild splenomegaly, and fluid within the swimbladder. In the immersion group, lethargy was the primary clinical sign and no mortalities were observed. In both experiments, the clinical signs appeared as early as two days post-infection and progressed in some cases, while in others, signs of healing were observed by the 8th day post-infection. *Flavobacterium* spp. were re-isolated from the brain, kidneys, ascites, and swimbladder fluid of dead and moribund fish. No *Flavobacterium* spp. were recovered from the kidneys or brains of fish surviving until the end of the experiment, nor from any of the negative control fish.

Histopathology

Microscopic changes observed in dead and moribund fish, as well as survivors following the 28 day observation period, included renal melano-macrophage hyperplasia, degeneration of kidney tubules, edema in the renal interstitial tissues, and proteinaceous casts within tubular lumens (Figure 3). Sagittal sections from the grossly affected caudal peduncles exhibited heterophilic cellulitis and myodegenerative

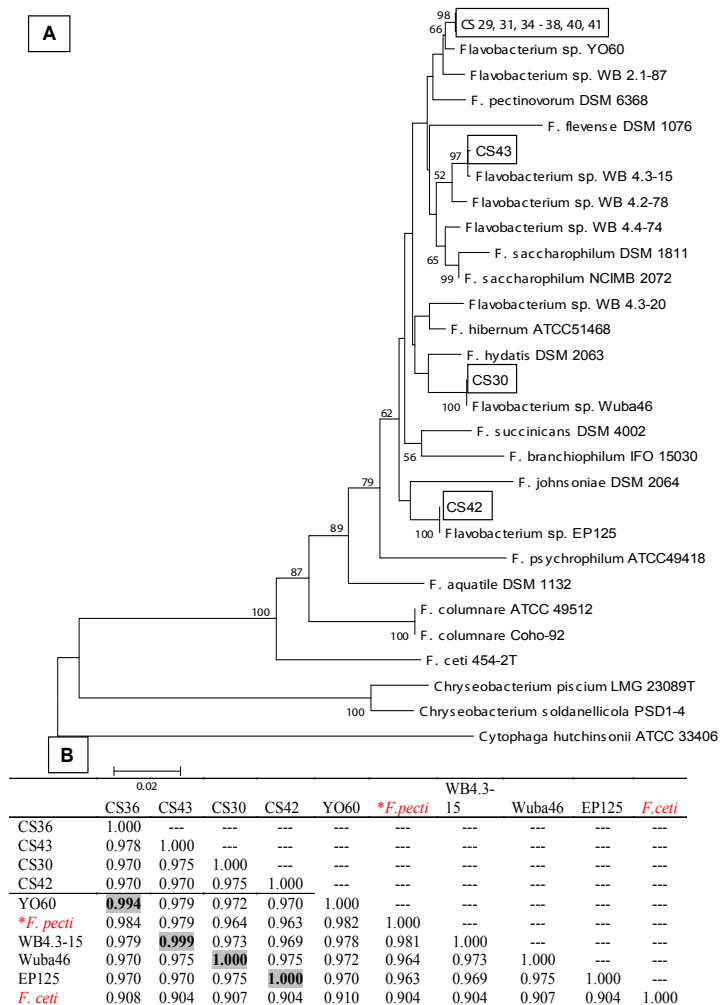


Figure 1: (A) Phylogenetic relationships of the 12 *Flavobacterium* isolates (CS29-31, 34-38, 40-43), retrieved from moribund coho salmon yolk sac and swim-up fry, identified by constructing a Neighbor-Joining tree with Kimura's 2-parameter model based on 676bp region of 16S rRNA sequences. The sequences were aligned in RDP. *Cytophaga hutchinsonii* ATCC 33406 was used as an out group. Bootstrap values greater than 50 were shown in the figure. (B) Similarity index table constructed based on sequence identity of 676bp. CS36 represents the clade of 9 isolates. The highest similarities of the 12 isolates are highlighted.

changes (Figure 4A and 4B). In infected fish suffering from ascites, a proteinaceous exudate was apparent in the coelomic cavity (Figure 4C). Fish in the negative control group did not exhibit any histopathological abnormalities.

Discussion

Flavobacterium spp. isolated in this study were retrieved from the brains and kidneys of moribund coho salmon fry that were tentatively diagnosed as suffering from EMS by hatchery personnel. Findings of this study, however, suggest that these *Flavobacterium* spp. are likely the etiologic agent of this mortality episode. This conclusion is based on a number of findings. First, the affected coho salmon fry at the Platte River State Fish Hatchery were prophylactically treated with a thiamine hydrochloride bath, a method that is considered very effective at combating the development of EMS [15-18]. Second, analysis of total thiamine contents in eggs and eyed eggs of 12 representative families of the same coho salmon spawning run averaged 3.48 ± 0.61 nmol/g (D. Holeyfield, unpublished data), meaning that thiamine was available in the eggs for the developing embryos. Third, *Flavobacterium* spp. were isolated from the brain and kidneys of nearly every moribund

fish, which indicates that the infection was both systemic and wide spread in affected fish, whereas no flavobacteria were recovered from the examined healthy fish. Last, experimental infection studies demonstrated that strain CS36, originally isolated from moribund coho salmon yolk sac fry, can cause morbidity and mortality in coho salmon fry. This is not the first time in which a *Flavobacterium* sp. has been associated with fry mortality of an *Oncorhynchus* sp. *Flavobacterium psychrophilum* causes heavy losses in the early life stages of rainbow trout (*Oncorhynchus mykiss*), which can be difficult to control [14], and are collectively referred to as rainbow trout fry syndrome [13]. In this study, no *F. psychrophilum* was isolated from healthy or diseased fish; rather *Flavobacterium* spp., that have never before been reported as causative agents of early life stage mortalities in fish, are the likely etiologic agents.

Biochemical, morphological, and 16S rDNA sequence investigations identified the 12 representative isolates retrieved from the brains and kidneys of moribund coho salmon as *Flavobacterium* spp. Within the genus *Flavobacterium*, the coho salmon strains clustered with many fish pathogenic *Flavobacterium* spp. (such as

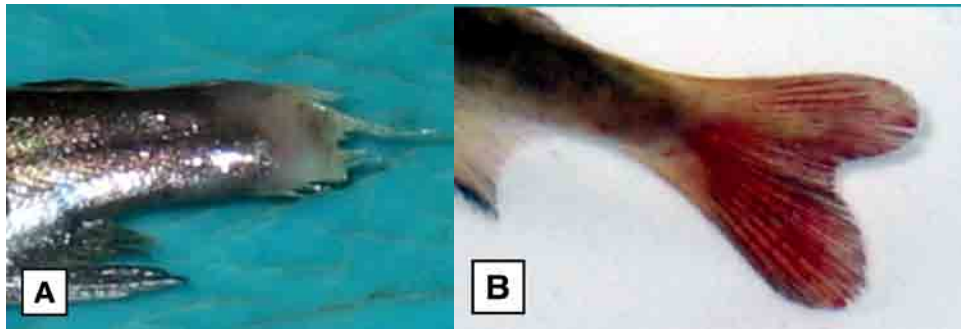


Figure 2: Coho salmon (*Oncorhynchus kisutch*) fry (3 months old) intraperitoneally infected with the *Flavobacterium* sp. CS36 strain with (A) extensive caudal fin erosion and necrosis that has progressed into the muscle of the caudal peduncle. (B) severe erythema in the caudal fin rays.

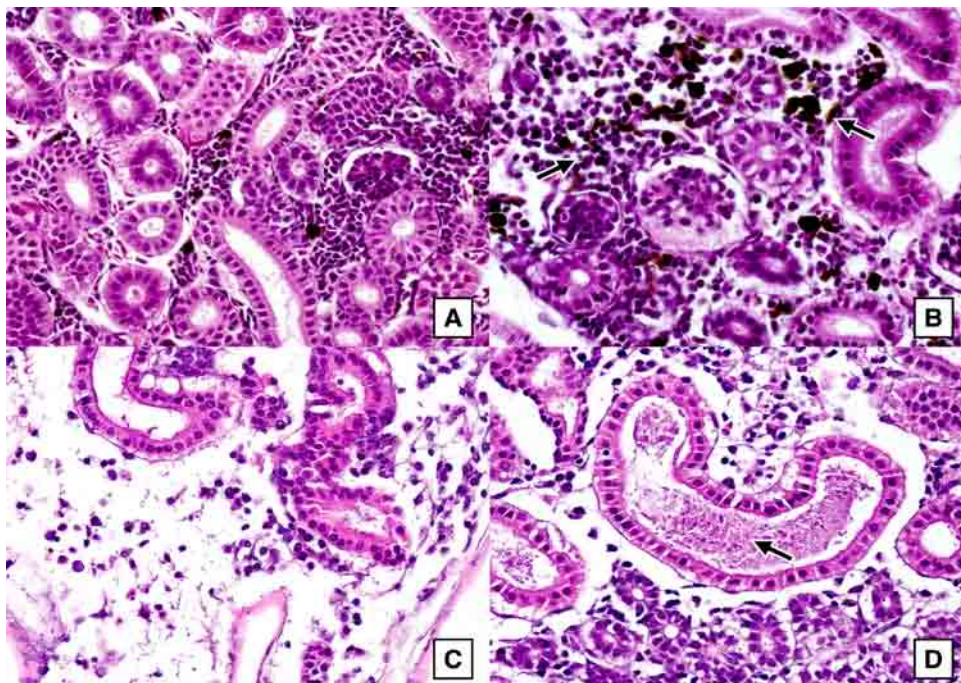


Figure 3: Sections of kidney tissues of coho salmon fry (three months old) stained with H&E and magnified 400X showing: (A) healthy kidney tissues of coho salmon from the negative control group. (B) kidney tissues of an intraperitoneally infected fish with melanomacrophage hyperplasia, (C) kidney tissues of an intraperitoneally infected fish with tubular degenerative changes and edema within the renal interstitium, (D) kidney tissues of an intraperitoneally infected fish with renal tubular degeneration and proteinaceous casts in the tubular lumen (arrow)

YO60, *F. saccarophilum*, *F. hydatis*, *F. succinicans*, *F. branchiophilum*, *F. johnsoniae* and *F. psychrophilum*) [10,12] as well as a number of recently isolated environmental strains from Europe with unknown pathogenicity to fish [30-32]. These findings are interesting in that they suggest that multiple *Flavobacterium* spp. can act synergistically during disease outbreaks in fish and that coho salmon fry are vulnerable to other *Flavobacterium* spp. that have not previously been associated with such syndromes in salmonids.

Phylogenetic analysis, along with cultural, morphological, and biochemical characterization, provided evidence that nine of the 12 isolates belong to one phylotype that also dominated the coho salmon fry disease outbreak. This strain exhibited substantial sequence resemblance (99.4%) to the YO60 *Flavobacterium* sp. isolated from long fin eels in South Africa that proved pathogenic to fish by experimental

infection [12]. It also bears a striking resemblance to *Flavobacterium* sp. WB 2.1-87, a strain that was isolated from the freshwater creek, Westerhöfer Bach, Harz Mountains, Germany [31], and *F. pectinivorum* (98.4%), which is known for its ability to degrade complex biopolymers. Strain CS43 also clustered with 2 *Flavobacterium* sp. strains of the Westerhöfer Bach of no reported pathogenicity. Strain CS30 was also of interest despite its infrequent isolation from moribund coho salmon due to its 100% sequence similarity with *Flavobacterium* sp. strain Wuba46, which was isolated from a freshwater aquifer in Germany (GenBank AF336355), and due to its clustering with *F. hydatis*, which was first isolated and described from diseased fish in two Michigan hatcheries [33,34]. There are no reports on the pathogenicity of the Wuba46 isolate in fish, or on the EP125-*Flavobacterium* sp., isolated by O'Sullivan et al. [30] from epilithon samples from the surfaces of stones in the River Taff in Cardiff, UK. Both strains, however, bear

	CS30	CS36	CS37	<i>F. pectinovorum</i>	<i>F. hydatis</i>
Gliding Motility	+	+	+	+ ¹	+ ²
Flexirubin Type Pigment	+	+	+	+ ¹	+ ¹
Congo Red Absorption	-	-	-	- ²	- ²
Growth at 25°C	+	+	+	+ ²	+ ²
Glucose Utilization	-	-	-	+ ²	+ ²
TSI Reaction	K/N/-	K/N/-	K/N/-	•	•
Lysine Decarboxylase	-	-	-	•	- ¹
Ornithine Decarboxylase	-	-	-	•	- ¹
Arginine Dihydrolase	-	-	-	•	•
Phenylalanine Deaminase	+	-	-	•	- ¹
Esculin Hydrolysis	+	+	+	+ ²	+ ²
Cytochrome Oxidase	-	-	-	+ ¹	- ¹
Catalase	+	+	+	+ ¹	+ ¹
Lipase	+	-	-	+ ¹	+ ¹
Gelatinase	+	+	+	+ ¹	+ ¹
Caseinase	+	+	+	+ ²	+ ¹
Starch Hydrolysis	+	+	+	+ ²	+ ¹
Urease	-	-	-	- ¹	- ¹
Agar Degradation	-	-	-	- ¹	- ²
ONPG	+	+	+	+ ²	+ ²
Susceptibility to 0129	-	-	-	+ ¹	- ¹
Simmons Citrate	-	(+)	(+)	•	•
Production of Indole	-	-	-	- ¹	- ¹
Production of H ₂ S	-	-	-	- ¹	- ¹

Table 1: Biochemical characteristics of representative *Flavobacterium* spp. strains CS30, CS36 and CS37 recovered from coho salmon. Tests were inoculated with 24 hr old subcultures and incubated at 22°C for up to 7 days. Results are presented as + for positive reaction; - for negative reaction; and (+) for weak positive reactions. For the TSI reaction, results are expressed as K/N/- designating alkaline slant/nonreactive butt/no production of H₂S/ no production of gas. For comparative purposes, biochemical characteristics of both *Flavobacterium pectinovorum* and *F. hydatis* are included as reported by 1: Reichenbach (1989) and 2: Bernardet and Bowman (1996) or *: not reported

close resemblance to *F. johnsoniae*, which has been associated with fish disease [10].

The phylogenetic relationship between the four coho salmon fry phylotypes is <98%, suggesting that they are not identical strains of *Flavobacterium* as per the criteria set by Bernardet et al. [22] for the taxonomy of the family Flavobacteriaceae. The heterogeneity of coho salmon strains was further verified by extensive biochemical and enzyme comparative analyses performed on CS30 and CS36, which demonstrated that the two strains are phenotypically different. Both coho salmon strains exhibited strong protease activity on casein and/or gelatin substrates, indicating the presence of extracellular proteases that may enhance the virulence and/or disease progression in infected fish [13]. Both coho salmon strains were also different in their biochemical reactions when compared to common fish pathogenic flavobacteria, such as *F. johnsoniae*, *F. succinicans*, *F. saccharophilum*, *F. psychrophilum*, *F. columnare* and *F. branchiophilum* [35]. The mixed flavobacterial infection observed in the coho salmon fry case with four phylogenetically distinct strains of the same genus is rarely observed in bacterial diseases of vertebrates and deserves further bacteriological and epidemiological study.

Experimental infection studies with *Flavobacterium* sp. CS36 clearly demonstrate that it can cause morbidity and mortality in coho salmon fry. However, the clinical signs in the experimentally infected fish were not identical to those originally observed in moribund yolk sac and swim-up fry. In particular, the spinning swimming behavior was not present despite successful re-isolation of CS36 from the brains

of experimental fish. Moreover, mortalities were less than experienced with the spontaneously affected fry and many of the survivors showed signs of recovery and bacterial clearance. These differences may be related to the fact that experimental fish were several months older than the spontaneously infected fish, as older fish have a more completely ossified skull that may hinder penetration of bacteria into the brain. Moreover, yolk sac fry are often reared in incubators that are densely packed, a matter that can stress fish and enhance pathogen transmission. On the other hand, it has been frequently reported that, even with the most pathogenic *Flavobacterium* spp., experimental infections can run a much milder course than natural disease outbreaks [36,37]. Stressors under hatchery culture conditions and fluctuations in environmental factors are believed to be the cause of the more pronounced disease course observed in spontaneously infected fish [13,36,38].

The source of infection to the coho salmon fry is unknown. Prior to incubation, fertilized eggs received an iodophore bath, which is a very effective egg surface disinfectant known for its bactericidal effects [39,40]. Egg incubators in the Platte River State Fish Hatchery receive sand-filtered spring water and embryonated eggs and yolk sac fry receive multiple formalin baths. Therefore, the likelihood that *Flavobacterium* spp. were introduced to the incubators from external sources, though possible, is minimal. Another possibility is intraovum transmission, which has been demonstrated in *F. psychrophilum* infections by Brown et al. [41] and Cipriano [42]. Regardless of the route through which the coho salmon fry were infected, our results clearly demonstrate the presence of four *Flavobacterium* spp. strains in Michigan fishes that are remarkably similar to those previously recovered from South Africa and Europe. How these novel flavobacteria emerged in the Platte River Hatchery warrants further investigation.

Transcontinental spread of pathogens through the international trade of fish and their products is not uncommon in the globalization era [1]. For example, until the mid-1980s, *F. psychrophilum* was known only in North America, where it was originally isolated from coho salmon and rainbow trout [43]. It was then found in France [44],

Enzyme	CS30	CS36	CS37
Alkaline Phosphatase	+	+	+
Esterase	(+)	(+)	(+)
Esterase Lipase	+	+	+
Lipase	-	-	-
Leucinearylaminidase	+	+	+
Valinearylaminidase	+	+	+
Cysteinearylaminidase	+	+	+
Trypsin	(+)	-	-
α-chymotrypan	-	-	-
Acid Phosphatase	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+
α-galactosidase	+	-	-
β-galactosidase	-	+	+
β-glucuronidase	-	-	-
α-glucosidase	+	+	+
β-glucosidase	-	-	-
N-acetyl- β-glucosaminidase	+	-	-
α-mannosidase	-	-	-
α-fucosidase	-	-	-

Table 2: Enzyme activities of *Flavobacterium* strains CS30, CS36, and CS37 recovered from the brains of moribund coho salmon and tested by the API-ZYM kit. Results are recorded following incubation at 22°C for 24 hrs and presented as + for positive reaction; - for negative reaction; and (+) for weak positive reactions.



Figure 4: Sagittal section of a lesion on the caudal peduncle of a coho salmon that was intraperitoneally infected with the *Flavobacterium* sp. CS36 strain showing: (A) heterophilic cellulitis (arrow), (B) myodegeneration (arrows), and (C) proteinaceous exudate within the coelom. The three H&E stained sections are shown at 400X magnification.

Germany [45], and Japan [46], and has since been reported from all regions in the world involved in salmonid aquaculture [13]. Since *F. psychrophilum* strains from all over the world maintain an extremely low level of diversity [47], the international trade of fish eggs has been blamed for its now widespread geographic range. The genetic similarity between *Flavobacterium* strains retrieved from coho salmon fry in Michigan, long-fin eels in South Africa, and surface water in Europe paints a picture that is similar to the case of *F. psychrophilum*, yet in the opposite direction. Michigan water is the recipient of many intentional and non-intentional introductions of fish and shellfish. For example, Michigan imported brown trout (*Salmo trutta*) from Germany in 1830, coho salmon in 1969 [48], and was invaded by the zebra mussel (*Dreissena polymorpha*), a native of the Caspian Sea in Eurasia, in the mid-1980s [49]. These non-native species could have brought *Flavobacterium* spp. with them; unfortunately, there are no baseline data on the diversity of *Flavobacterium* spp. present in fish or the aquatic environment in Michigan or the Laurentian Great Lakes basin prior to the settlement of invasive species. Prevention of the spread of these emerging flavobacteria to aquaculture facilities, other areas in the Great Lakes, or other aquatic ecosystems in North America should be a high managerial priority. Additionally, the findings of this study underscore the complexity of the etiology of mortalities in fish in their early life stages, a matter that requires caution in diagnosis so as to choose an effective control strategy.

Acknowledgements

We are indebted to personnel at the Platte River State Fish Hatchery, Michigan Department of Natural Resources, for their help in sample collection. The authors would also like to thank the United State Department of Agriculture-APHIS (10-9100-1293-GR) and the Great Lakes Fishery Trust (2010-1147) for funding this research.

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This article was originally published in a special issue, **Current and Emerging Diseases/Disorders of Fish in Aquaculture** handled by Editor(s). Prof. Patrick T.K. Woo, University of Guelph, Canada; Dr. Kenneth D. Cain, University of Idaho, USA.