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Deciphering the biodiversity of fish-pathogenic *Flavobacterium* spp. recovered from the Great Lakes basin

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ABSTRACT: Flavobacterial diseases negatively impact wild and cultured fishes worldwide. We recently reported on the presence of a large and diverse group of flavobacteria, many of which were associated with lesions in a number of Great Lakes fish species. Herein, we report on the characterization of 65 fish-associated *Flavobacterium* spp. isolates using 16S rRNA gene sequence analysis and phylogenetic analyses based upon neighbor-joining and Bayesian methodologies. Thirteen isolates were identified as the newly described fish-associated *F. plurextorum*, *F. spartansii*, and *F. tructae*, while 3 isolates were similar to *F. frigidimaris*; however, the remaining *Flavobacterium* spp. isolates did not conclusively match any described *Flavobacterium* spp. and thus were suspected as comprising novel flavobacterial species. A more comprehensive polyphasic characterization was undertaken on 6 isolates, representing a range of association with disease signs in hatchery-raised or free-ranging fish and genetic distinctness. Polyphasic characterization included physiological, morphological, and biochemical analyses, as well as additional phylogenetic analyses based upon near-complete sequencing of the 16S rRNA gene. Our findings demonstrated that that at least 5 of the 6 isolates are most likely novel species within the genus *Flavobacterium* that have never before been reported from fish. Pilot experimental challenge studies suggested that some of these *Flavobacterium* spp. can cause pathological lesions in fish and were re-isolated from the brains, spleens, livers, and kidneys of experimentally infected fish. The findings underscore the growing number and heterogeneity of flavobacteria now known to be capable of infecting fish.

KEY WORDS: *Flavobacterium* · Fish disease · Bacteria · Bacterial coldwater disease · Great Lakes

INTRODUCTION

Flavobacterial diseases are a significant source of mortality in wild and cultured fishes worldwide that have historically been attributed to 3 species within the genus *Flavobacterium* (Family *Flavobacteriaceae*); namely, *F. psychrophilum*, the etiological agent of bacterial coldwater disease (Borg 1960, Holt 1987) and rainbow trout fry syndrome (Madsen & Dalsgaard 1998); *F. columnare*, the causative agent of columnaris disease (Shotts & Starliper 1999); and *F. branchiophilum*, the putative agent of bacterial gill disease (Wakabayashi et al. 1989). On occasion, other *Flavobacterium* spp. have also been recovered from diseased fishes, including *F. succinicans* (Anderson & Ordal 1961), *F. johnsoniae* (Christensen 1977), *F. hydatis* (Strohl & Tait 1978), as well as other uncharacterized yellow-pigmented bacteria (reviewed in Austin & Austin 2007). Flavobacterial disease outbreaks manifest along a continuum of acute to

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chronic forms (Shotts & Starliper 1999, Austin & Austin 2007) and can be associated with considerable economic losses.

The genus *Flavobacterium* has rapidly expanded from 26 published species in 2006 (Bernardet & Bowman 2006) to over 100 published and proposed species at the time this manuscript was written (www.ezbiocloud.net/eztaxon; Kim et al. 2012). In a similar fashion, the number of *Flavobacterium* spp. being reported in association with diseased fish is also growing, as evidenced by the multiple novel *Flavobacterium* spp. that have been recovered and described from diseased fish being farmed in South America (e.g. Kämpfer et al. 2012) and Europe (e.g. Zamora et al. 2012, 2013, 2014). Likewise, studies conducted in our laboratory revealed that numerous *Flavobacterium* spp. were associated with both wild and farmed diseased fishes residing within the Great Lakes basin of North America (Loch et al. 2013). While a portion of the isolates were identified as *F. psychrophilum*, *F. columnare*, and *F. oncorhynchi*, the vast majority were distinct from the described *Flavobacterium* spp. Indeed, 2 novel flavobacterial taxa were described as a result of these studies (Loch & Faisal 2014a,b) and others are also suspected as being novel *Flavobacterium* spp.

Thus, the present study was undertaken to (1) characterize these potentially novel *Flavobacterium* spp.; (2) perform an in-depth polyphasic characterization on representatives of the isolated *Flavobacterium* spp.; and (3) shed light on the potential pathogenicity of the fully characterized *Flavobacterium* spp. under controlled laboratory conditions.

MATERIALS AND METHODS

Fish and sampling

The *Flavobacterium* spp. isolates of this study were retrieved from 17 fish species that were either cultured in hatcheries or collected from lakes and streams for fish health surveillance. Fish sampling and bacterial isolations were performed as described in Loch et al. (2013). Host species, date of isolation, and organ from which bacteria were originally recovered are detailed in Table 1.

Bacterial isolates

Flavobacterium spp. isolates (n = 65) were maintained in Hsu-Shotts broth (HSB; Bullock et al. 1986) or cytophaga broth (Anacker & Ordal 1955) supplemented with 20% (v/v) glycerol at −80°C, and revived for analysis in this study.

16S rRNA gene amplification and phylogenetic analysis

Genomic DNA extraction, amplification of the 16S rRNA gene, and gene sequencing were carried out for the 65 revived *Flavobacterium* spp. isolates as described in Loch et al. (2013), with the exception that sequencing was performed bi-directionally using the forward and reverse primers. Resultant sequences were compared with all formally described and 'candidate' *Flavobacterium* spp. that were downloaded from the National Center for Biotechnology Information database and the EzTaxon-e database (Kim et al. 2012) using the alignment tool available in the nucleotide Basic Local Alignment Search Tool software. Sequence alignment and neighbor-joining analysis was performed using Molecular Evolutionary Genetics Analysis software (MEGA; version 5.0), whereby evolutionary distances were calculated by the maximum composite likelihood method and topology robustness was evaluated by bootstrap analysis (n = 10 000 resamplings). In addition, Bayesian analysis was conducted in MrBayes 3.2 (Ronquist et al. 2012) using the general time reversible model. The Markov chains (n = 12) were run for up to 10 million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.05. In the instance where an average standard deviation of split frequencies of <0.05 was not achieved, a plot of the generation versus the log likelihood values was examined for stationarity and the potential scale reduction factor was confirmed to approach 1.00 (Ronquist et al. 2012). Two independent analyses were conducted, with the initial 25% of Markov chain Monte Carlo samples being discarded as burnin and sampling occurring every 1000 generations. Results from Bayesian analyses were visualized in FigTree v1.3.1 (Rambaut 2009).

Polyphasic characterization of representative *Flavobacterium* spp. isolates

The taxonomic status of 6 *Flavobacterium* spp. isolates (T91, T75, T18, S87, S21, and T76) was further investigated using a polyphasic approach. These 6 isolates were selected because of their dissimilarity

Table 1. Sixty-five Michigan *Flavobacterium* spp. isolates selected for 16S rDNA sequence and phylogenetic analyses in this study, as well as the fish species and organs from which they were originally recovered, and the date of isolation. W: wild fish; H: hatchery fish

Isolate ID	Accession no.	Closest relative (% 16S rDNA similarity)	Fish host species	Date of recovery	Organ of origin
S12	JX287884	*F. tructae* (99.5)	Chinook salmon (H)	05/2005	Gills
S21	JX287885	*F. aquidurense* (98.1)	Rainbow trout (H)	08/2005	Ulcer
S87	JX287883	*F. resistens* (97.9)	Largemouth bass (W)	08/2005	Kidney
T1	JX287787	*F. frigidimaris* (97.8)	Bluegill (W)	06/2005	Gills
T2	JX287788	*F. chilense* (98.7)	Lake whitefish (W)	11/2005	Kidney
T3	JX287789	*F. chilense* (98.8)	Lake whitefish (W)	11/2005	Kidney
T6	JX287791	*F. hercynium* (98.8)	Lake whitefish (W)	05/2006	Swim bladder
T8	JX287793	*F. saccharophilum* (98.6)	Brown trout (H)	11/2006	Swim bladder
T9	JX287794	*F. saccharophilum* (98.6)	Brown trout (H)	11/2006	Kidney
T10	JX287795	*F. plurextorum* (98.5)	Brown trout (H)	05/2007	Brain
T13	JX287796	*F. spartansii* (99.6)	Chinook salmon (W)	10/2007	Kidney
T14	JX287797	*F. tructae* (99.8)	Chinook salmon (W)	10/2007	Kidney
T15	JX287798	*F. plurextorum* (98.3)	Chinook salmon (W)	10/2007	Kidney
T17	JX287800	*F. aquidurense* (98.4)	Steelhead (W)	04/2008	Kidney
T18	JX287801	*F. hydatis* (98.7)	Brown trout (H)	05/2008	Gills
T21	JX287804	*F. hydatis* (98.5)	Brown trout (H)	05/2008	Gills
T23	JX287805	*F. plurextorum* (99.8)	Steelhead (H)	06/2008	Gills
T25	JX287806	*F. plurextorum* (99.9)	Steelhead (H)	06/2008	Gills
T27	JX287808	*F. chungangense* (98.2)	Brook lamprey (W)	06/2008	Kidney
T37	JX287810	*F. tructae* (98.4)	Mottled sculpin (W)	09/2008	Gills
T47	JX287811	*F. chungangense* (98.4)	Brown trout (W)	09/2008	Gills
T54	JX287815	*F. piscis* (98.3)	Brown trout (H)	10/2008	Gills
T56	JX287816	*F. tiangeerense* (97.8)	Coho salmon (W)	10/2008	Kidney
T57	JX287817	*F. saccharophilum* (98.2)	Coho salmon (W)	10/2008	Swim bladder
T59	JX287818	*F. plurextorum* (99.9)	Brook trout (H)	01/2009	Kidney
T61	JX287819	*F. hercynium* (98.7)	Brown trout (H)	02/2009	Fins
T65	JX287820	*F. hercynium* (98.1)	Walleye (W)	04/2009	Kidney
T66	JX287821	*F. hercynium* (98.4)	Chinook salmon (H)	03/2009	Kidney
T69	JX287822	*F. saccharophilum* (98.9)	Steelhead (W)	04/2009	Kidney
T70	JX287823	*F. hercynium* (98.5)	Lake herring (H)	05/2009	Kidney
T71	JX287824	*F. hercynium* (98.3)	Lake herring (H)	05/2009	Kidney
T73	JX287825	*F. hercynium* (98.4)	Lake herring (H)	05/2009	Kidney
T74	JX287826	*F. degerlachei* (97.4)	Brown trout (H)	06/2009	Kidney
T75	JX287827	*F. tiangeerense* (97.5)	Brown trout (H)	06/2009	Kidney
T76	JX287828	*F. pectinovorum* (98.4)	Rainbow trout (H)	06/2009	Kidney
T77	JX287829	*F. pectinovorum* (98.0)	Rainbow trout (H)	06/2009	Kidney
T91	JX287833	*F. anhuiense* (98.2)	Channel catfish (H)	07/2009	Kidney
T92	JX287834	*F. anhuiense* (97.8)	Channel catfish (H)	07/2009	Kidney
T93	JX287835	*F. frigidimaris* (98.8)	Brook trout (H)	08/2009	Gills
T95	JX287836	*F. plurextorum* (99.9)	Brown trout (H)	08/2009	Gills
T96	JX287837	*F. plurextorum* (99.9)	Brown trout (H)	08/2009	Gills
T99	JX287838	*F. frigidimaris* (99.1)	Lake trout (H)	08/2009	Gills
T100	JX287839	*F. frigidimaris* (98.9)	Lake trout (H)	08/2009	Gills
T101	JX287840	*F. saccharophilum* (98.7)	Chinook salmon (H)	03/2009	Kidney
T102	JX287841	*F. saccharophilum* (98.7)	Brook trout (H)	02/2009	Kidney
T103	JX287842	*F. plurextorum* (99.8)	Sea lamprey (W)	05/2004	Kidney
T108	JX287845	*F. pectinovorum* (98.5)	Brown trout (H)	09/2009	Fins
T123	JX287857	*F. tructae* (99.6)	Lake trout (H)	02/2010	Kidney
T124	JX287858	*F. tructae* (99.6)	Lake trout (H)	02/2010	Kidney
T129	JX287860	*F. succinicans* (98.0)	Brook trout (H)	03/2010	Kidney
T131	JX287861	*F. pectinovorum* (98.3)	Brook trout (H)	03/2010	Kidney
T132	JX287862	*F. hercynium* (98.8)	Walleye (W)	03/2010	Kidney
T141	JX287869	*F. limicola* (97.9)	Steelhead (W)	04/2010	Kidney
T142	JX287870	*F. limicola* (97.8)	Brook trout (W)	06/2010	Gills
T148	JX287871	*F. glacei* (97.9)	Brook trout (W)	06/2010	Gills
T151	JX287873	*F. hercynium* (98.6)	Brook trout (W)	06/2010	Gills
T156	JX287874	*F. succinicans* (98.5)	Brown trout (W)	06/2010	Gills
T157	JX287875	*F. aquidurense* (98.8)	Brown trout (W)	06/2010	Gills
T158	JX287876	*F. succinicans* (98.8)	Brown trout (W)	06/2010	Gills
T159	JX287877	*F. hydatis* (98.9)	Brown trout (W)	06/2010	Gills
T160	JX287878	*F. limicola* (98.1)	Brown trout (W)	06/2010	Gills
T161	JX287879	*F. succinicans* (98.5)	Brown trout (W)	06/2010	Gills
T164	JX287880	*F. plurextorum* (99.7)	Steelhead (H)	06/2010	Gills
T165	JX287881	*F. plurextorum* (99.8)	Steelhead (H)	06/2010	Gills
T166	JX287882	*F. plurextorum* (98.4)	Steelhead (H)	06/2010	Kidney

to other described *Flavobacterium* spp., association with systemic infections in their host and/or mortality episodes, and association with gross pathological changes in naturally infected hosts. Assays for polyphasic characterization were selected based upon those that are recommended for the description of novel bacterial taxa in general, and for members of the family *Flavobacteriaceae* in particular (Bernardet et al. 2002), which included the following: morphological, physiological, and biochemical characterization; antibiotic susceptibility testing; and phylogenetic analysis based upon near-complete 16S rDNA.

Morphological, physiological, and biochemical characterization

Isolates cultured for 24–48 h at 22°C were used during these analyses and all reagents were purchased from Remel unless noted otherwise. Isolates were assayed for the Gram reaction and catalase (3% H_2O_2) activity, and the presence of a flexirubin-type pigment (3% KOH) and cell wall-associated galactosamine glycans (0.01% w/v Congo red solution; Bernardet et al. 2002). Motility was assessed in sulfur indole motility (SIM) deeps and gliding motility via the hanging-drop technique (Bernardet et al. 2002). Additional characterization included colony morphology on cytophaga agar (CA), growth on cetrimide and nutrient agars (Sigma-Aldrich), marine agar (Becton Dickinson Microbiology Systems), trypticase soy agar (TSA), and MacConkey agar; growth on Hsu-Shotts medium (HSM) at 4, 15, 22, 37, and 42°C; growth on HSM plates at salinities ranging from 0 to 5.0% in 1% increments; acid/gas from glucose and acid from sucrose (1% final concentration, phenol red broth base); triple sugar iron (TSI) reaction; hydrolysis of esculin (bile esculin agar); use of citrate as a sole carbon source (Simmon's citrate); production of indole and/or hydrogen sulfide on SIM medium; lysis of hemoglobin (0.1% w/v) and degradation of collagen (0.1% w/v), casein (5% w/v), and elastin (0.5%) as modified from Shotts et al. (1985) using HSM as the basal medium; activity for gelatinase (Whitman 2004), phenylalanine deaminase (Sigma-Aldrich), and DNase; activity for alginase (5% w/v alginic acid, Sigma-Aldrich, in HSM), pectinase (5% w/v pectin from apple, Sigma, overlay), chitinase (5% w/v chitin from crab shells, Sigma), and carboxymethylcellulase (0.15% w/v, Sigma-Aldrich, overlay), all modified from Reichenbach (2006) with HSM as basal medium; activity for chondroitin sulfatase C (0.2% w/v chondroitin sulfate sodium salt from shark cartilage, Sigma-Aldrich, HSM basal medium) and amylase (as modified from Lin et al. 1988; using HSM as basal medium); degradation of Tween 20 and Tween 80 (1% v/v, Sigma-Aldrich); brown pigment production from L-tyrosine (0.5% w/v, Sigma-Aldrich), modified from Pacha & Porter (1968) using HSM as basal medium; and degradation of agar on TSA. When HSM was used as the basal medium, gelatin and neomycin were not added. Commercially available identification galleries (i.e. API 20E, API 20NE, API ZYM, and API 50CH; BioMerieux) were inoculated according to the manufacturer's protocol; however, tests were incubated at 22°C and read from 24 h post-inoculation up until 7 d, with the exception of the API ZYM, which was read at 72 h.

Antibiotic susceptibility testing

Flavobacterium spp. isolates were tested for antibiotic susceptibility using the Kirby-Bauer disk diffusion method. Cultures grown on HSM (24–48 h) were resuspended in sterile 0.85% saline and adjusted to an optical density of 0.5 at 600 nm in a Biowave CO8000 Cell Density Meter (WPA). The bacterial suspension (1 ml) was inoculated onto dilute Mueller-Hinton agar (Hawke & Thune 1992) without 5% calf serum in duplicate. Antibiotic-imbibed disks were placed onto the medium and plates were incubated at 22°C for 24 to 48 h, at which time the zones of inhibition were measured. Antibiotics included polymyxin-B (300 IU), oxytetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), erythromycin (15 µg), ampicillin (10 µg), florfenicol (30 µg), penicillin G (10 IU), and the vibriostatic agent 0/129 (2,4-diamino,6,7-di-isopropyl pteridine; 10 µg).

Phylogenetic analyses based on near-complete 16S rDNA

PCR amplification was conducted using the universal primers 8F (5′-AGT TGA TCC TGG CTC AG-3′) and 1492R (5′-ACC TTG TTA CGA CTT-3′; Sacchi et al. 2002) and phylogenetically analyzed as described above. However, primers 8F, 1492R, 518F (5′-TAC CAG GGT ATC TAA TCC-3′), 800R (5′-CCA GCA GCC GCG GTA ATA CG-3′), and 1205F (5′-AAT CAT CAC GGC CCT TAC GC-3′) were used for sequencing. In addition, Bayesian analysis was conducted in MrBayes 3.2 as described above, with the exception that 8 chains were run in 4 independent analyses.

Ability of representative *Flavobacterium* spp. isolates to induce pathological lesions in fish

The experimental challenge studies were conducted in accordance with the Michigan State University Institutional Animal Care and Use Committee.

Fish

Disease-free certified, 1-mo post-hatch Chinook salmon *Oncorhynchus tshawytscha*, brook trout *Salvelinus fontinalis*, and brown trout *Salmo trutta* were allowed to acclimate to laboratory conditions for 8 wk before use in experimental challenges at the University Research Containment Facility at Michigan State University. Fish were fed Bio Vita trout feed (Bio-Oregon) to apparent satiation and maintained in aerated flow-through tanks (~400 l; 12-h photoperiod) with dechlorinated pathogen-free water at a temperature of 10±1°C. All tanks were cleaned daily. Prior to the experiment, subsets of all fish species were analyzed for the presence of non-fastidious bacteria, *Renibacterium salmoninarum*, flavobacteria, parasites, and viruses that replicate in EPC (epithelioma papulosum cyprini) and CHSE-214 (Chinook salmon embryo) cell lines.

Determination of growth kinetics for flavobacterial strains

One 48-h-old colony forming unit (cfu) from each flavobacterial isolate was inoculated into 40 ml HSB supplemented with 5% (v/v) horse serum and 0.02% (v/v) mineral solution of Lewin and Lounsberry (Michel et al. 1999) and incubated statically at 22°C. Immediately after inoculation (Time 0) and at 8, 24, 48, 72, 96, 120, 144, and 168 h post-inoculation, the bacterial suspension was gently vortexed and 2 ml removed for optical density determination at 600 nm in a Biowave CO8000 Cell Density Meter and colony enumeration via plate counts.

Experimental challenge via intraperitoneal injection

Prior to experimental challenge, each *Flavobacterium* spp. isolate was passaged in Chinook salmon. Briefly, ~10^8 cfu were injected intraperitoneally (IP) into 3 Chinook salmon per isolate as described below. Fish were then euthanized 3 d post-infection and bacterial cultures taken from the kidneys on enriched HSM. Bacterial growth was then subcultured, the purity was verified, and freezer stocks were prepared. In addition, recovered isolates were identified via gene sequencing and phylogenetic analysis.

Chinook salmon (mean ± SD weight 14.0 ± 6.0 g; mean ± SD length 11.5 ± 1.9 cm), brook trout (mean weight 11.3 ± 4.2 g; mean length 10.9 ± 1.5 cm), and brown trout (mean weight 3.1 ± 0.9 g; mean length 7.0 ± 1.5 cm) were first anesthetized in carbonate-buffered tricaine methanosulphonate (MS-222; n = 5 per isolate per fish species) at a concentration of 100 mg l^{-1} and then IP injected with 100 µl of a bacterial suspension containing 7.3 × 10^7 to 7.0 × 10^8 cfu of flavobacteria. Control fish (n = 5) were injected with 100 µl of sterile phosphate-buffered saline. Groups of challenged fish and negative control fish were immediately placed in their own well aerated, flow-through PVC tanks (70 l) receiving 1.26 l min^{-1} and monitored for 14 d, at which time survivors were euthanized. Fish were checked twice daily for morbidity/mortality and fed daily. In the event that severe signs of morbidity were observed, the affected fish was euthanized with an overdose of MS-222 and immediately necropsied. Gross examinations were performed on all fish, and liver, spleen, kidney, and brain samples were collected and inoculated directly onto enriched HSM (incubated at 22°C) and CA (incubated at 15°C) plates and incubated for up to 7 d. Representative isolates recovered from challenged fish in each experiment were identified via gene sequencing and phylogenetic analysis as described previously to confirm their original identities.

RESULTS

Phylogenetic analysis based upon the 16S rRNA gene was capable of differentiating the vast majority of the described *Flavobacterium* spp. According to 16S rRNA gene sequence analysis (sequence length ranging from 1337 to 1380 bp), only 13 of the 65 flavobacterial isolates conclusively matched a described *Flavobacterium* spp. (Fig. S1 in the Supplement at www-int-res.com/articles/suppl/d112p045_supp.pdf). Isolates T164, T25, T165, T103, T23, T96, T59, and T95 were 99.7–99.9% similar to *F. plurextorum* (Table 1) and formed a robustly supported clade with the type strain (Fig. S1), while isolates T13, S12, T14, T124, and T123 were 99.5–99.8% similar to *F. spartansii* and *F. tructae*. In addition, isolates T99, T100, and T93 were 98.8–99.1% similar to the *F. fri-*

gidimaris reference sequence (Table 1) and clustered with the type strain (Fig. S1). The remaining flavobacterial isolates did not conclusively match any of the described *Flavobacterium* spp., whereby 44 isolates fell into 17 well-supported clusters (i.e. bootstrap value/posterior probability ≥70%) that were distinct from all described *Flavobacterium* spp. (Fig. S1), while the topology for 5 isolates could not be resolved.

To investigate the distinctness of these fish-associated flavobacteria, polyphasic characterization analyses were undertaken for 6 isolates that belonged to 6 distinct taxonomic clusters (i.e. T91, T75, T18, S87, S21, and T76), the results of which are detailed below.

Sequence analysis of the near-complete 16S rRNA gene found isolate T91 to be most similar to *F. anhuiense* (98.2%) and *F. ginsenosidimutans* (97.8%), while T75 was most similar to *F. tiangeerense* (97.5%) and *F. frigidarium* (97.4%). Isolate T18 was most similar to *F. hydatis* (98.7%) and *F. oncorhynchi* (98.2%), while S87 was most similar to *F. resistens* (97.9%) and *F. oncorhynchi* (97.8%). Lastly, S21 was most similar to *F. aquidurense* (98.1%) and *F. frigidimaris* (98.0%), while T76 was most similar to *F. pectinovorum* (98.4%) and *F. hydatis* (98.1%).

Phylogenetic analyses (based upon the near-complete 16S rRNA gene sequence) using Bayesian and neighbor-joining methodologies showed that the topologies of the resultant dendrograms were identical at some nodes (depicted in Fig. 1 as nodes with a black circle), but Bayesian analysis was able to predict well-supported relationships for some of the Michigan fish-associated taxa whereby neighbor-joining analysis could not. For example, *Flavobacterium* sp. T91 fell into a clade with a bootstrap value <70% in neighbor-joining analysis, but was supported as being distinct according to Bayesian analysis (posterior probabilities of 0.95; Fig. 1). Similarly, *Flavobacterium* sp. T18 was well supported as being distinct (posterior probability of 0.97; Fig. 1), as was isolate S87 (posterior probability of 0.94; Fig. 1). However, the topology for *Flavobacterium* spp. T75, T76, and S21 was unresolved (i.e. boot strap value and posterior probability <70) using both phylogenetic methodologies (Fig. 1).

When grown on CA, T91, T18, S87, S21, and T76 produced colonies that were yellow, semi-translucent, and nearly flat with irregular spreading margins, while T75 produced colonies that were yellow, semi-translucent, and low convex with entire margins. The physiological and biochemical assay results that varied amongst the isolates are presented in Table 2, while results that were uniform amongst the 6 isolates are presented in the legend of Table 2. As displayed in Tables S1–S6 in the Supplement (at www.int-res.com/articles/suppl/d112p045_supp.pdf), all 6 Michigan flavobacterial isolates were distinct from their 2 closest relatives, in a number of biochemical and physiological characteristics. Antibiotic sensitivity testing based upon the disk diffusion method revealed that all 6 flavobacterial isolates were sensitive to florfenicol, 5 of 6 were resistant to trimethoprim-sulfamethoxazole, polymyxin-B, O129, and penicillin G, while sensitivities to the remaining antibiotics varied amongst the isolates (Table 3).

Growth kinetic experiments revealed that the 6 flavobacterial isolates were in a logarithmic to late-logarithmic growth phase at ~24 h post-inoculation at 22°C. Hence, 18–24 h broth cultures were selected for use in experimental challenges. Analysis performed on experimental fish prior to their use showed the absence of flavobacteria and other pathogens in their internal organs.

The percent mortality resulting from each of the experimental challenges, during which fish were IP injected with 7.3×10^7 to 7.0×10^8 cfu, varied between 0 and 40% amongst the isolates (Table 4). For example, mortality totaled 27% across all 3 salmonid species challenged with isolate S21, 13% in fish challenged with isolate T76, and 7% in fish challenged with isolate T91 (Table 4), whereby mortalities occurred between 1 and 4 d post-infection. No mortalities occurred in fish challenged with T18, T75, or S87, or in the control fish. Among fish mortalities, flavobacteria were recovered from the livers, spleens, kidneys, and brains of the infected fish. In fish surviving until 14 d post-infection, isolates T91, S21, and T76 were recovered from all 4 organs in some of the challenged fish, while isolate S87 was recovered from the livers, spleens, and kidneys only. However, T75 was recovered from one kidney of a challenged brown trout, and T18 was not recovered from any organs of the 15 challenged fish. In all cases, bacteria recovered from experimentally challenged fish were identified as the original bacterial species utilized in the injection according to 16S rDNA sequencing and phylogenetic analysis. *Flavobacterium* spp. were not recovered from any control fish during the course of this study.

Gross pathology in fish challenged with S21 varied, whereby the most severe signs included unilateral exophthalmia and concurrent periocular hemorrhage (Fig. 2A), mottled external coloration, gill

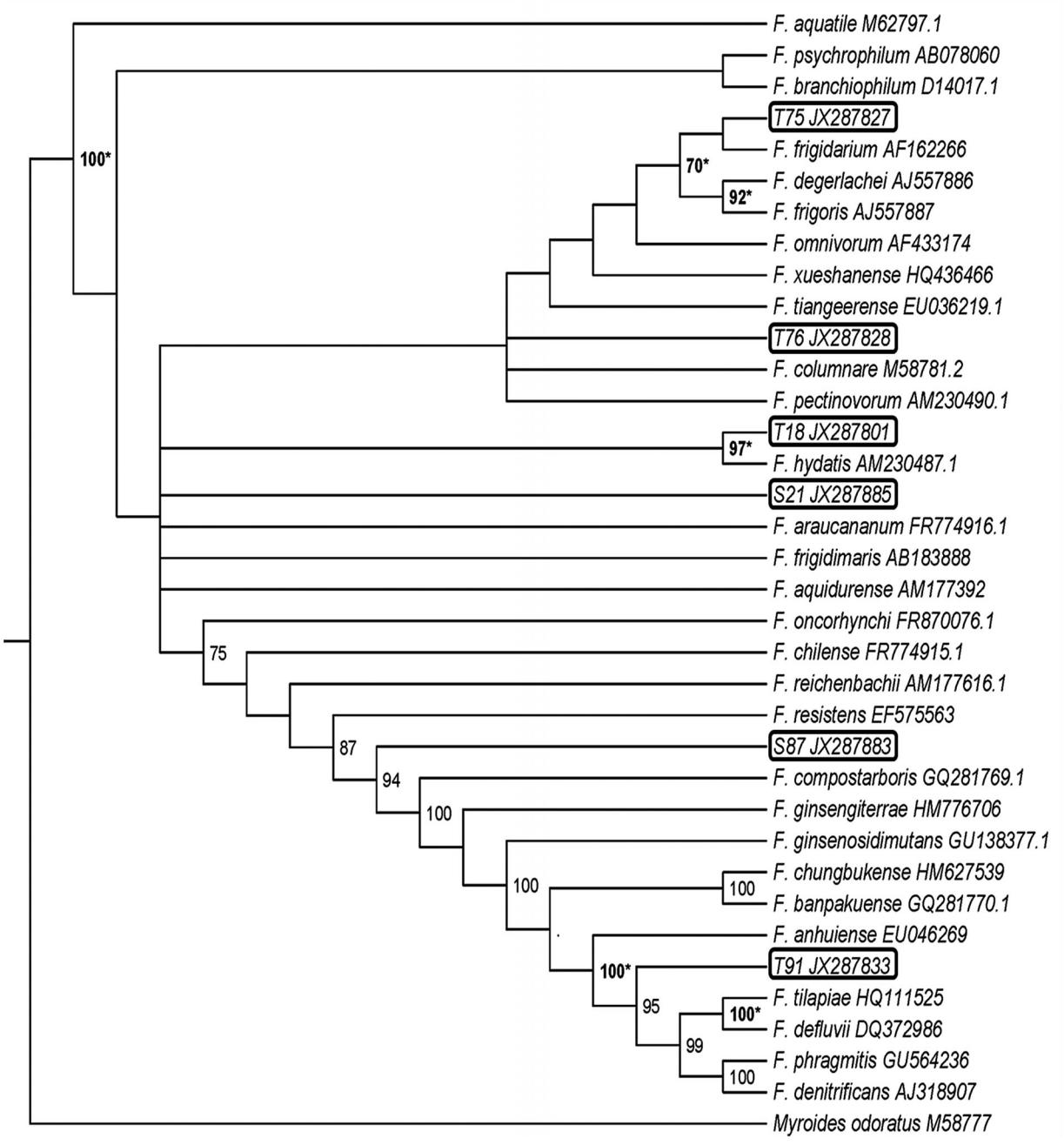

Fig. 1. Cladogram depicting the relationships of *Flavobacterium* spp. T91, T75, T18, T76, S21, and S87 (black rectangles) generated using Bayesian analysis with the general time reversible model and gamma-shaped rate variation with a proportion of invariable sites Bayesian in MrBayes 3.2. The Markov chain was run for up to 10 million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.05%. Four independent analyses were conducted, both with 1 cold and 3 heated chains using the default heating parameter (temp = 0.2). The initial 25% of Markov Chain Monte Carlo samples were discarded as burn-in and sampling occurred every 1000 generations. Bolded posterior probability values with a star denote that the same node was supported (i.e. bootstrap value ≥70%) in neighbor-joining analyses

pallor, flared opercula, swelling/pallor/congestion/friability of the liver, swollen and enlarged spleen, swim bladder hemorrhage (Fig. 2B), swelling/edema/congestion/pallor of the kidney, and focal petechial hemorrhage within the brain. Pathological changes in fish challenged with T76 also varied and included gill pallor, mottled external coloration, hepatic congestion/hemorrhage, fibrinous adhesions

Table 2. Characteristics that were variable among 6 Michigan fish-associated *Flavobacterium* spp. isolates examined in this study. +: positive test result; (+): weak positive test result; −: negative test result; NG: no growth. All 6 isolates were Gram-negative rods that were nonmotile in sulfur-indole-motility deeps, but 5 of 6 were motile via gliding (T75 was unable to glide). All isolates possessed a flexirubin-type pigment and grew on trypticase soy agar (T75 with weak growth), nutrient agar, and Hsu-Shotts Medium, but did not grow on MacConkey and cetrimide agars. The 6 isolates grew from 4 to 22°C but not at 42°C, and at salinities of 0–1% but not at 3–5%. All 6 isolates produced an alkaline slant and no reaction in the butt in triple sugar iron without hydrogen sulfide or gas, hydrolyzed esculin (weakly for T18), and utilized citrate as a sole carbon source. None of the isolates produced indole, alginase, collagenase, chitinase, or carboxymethyl cellulase, nor did they degrade agar or Tween 80. However, all 6 isolates produced catalase, caseinase, pectinase, and amylase. On the API 20E, none of the isolates had arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, or tryptophan deaminase activities, nor did they produce acid from mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, or arabinose. On the API 20NE, the isolates degraded para-nitrophenyl-β-D-galactopyranoside and assimilated D-mannose and D-glucose, while none were able to assimilate D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, or phenylacetic acid. On the API ZYM, all isolates were positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, napthol-AS-BI-phosphohydrolase, α-glucosidase, and N-acetyl-β-glucosaminidase activities, but did not show lipase or α-mannosidase activities. On the API 50CH (using CHB/E medium), none of the isolates produced acid from glycerol, erythritol, D-ribose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, D-melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, or potassium 5-ketogluconate

Characteristic	T91	T75	T18	S87	S21	T76
Cell size (µm)	2–10	1.5–3.0	1.0–2.0	1.0–2.5	2.0–4.0	2.0–4.0
Congo red absorption	(+)	−	−	−	+	+
Growth on marine agar	+	+	−	−	−	+
Growth at 37°C	−	−	−	(+)	−	−
Growth at 2% salinity	(+)	−	−	−	+	+
Nitrate reduction	+	−	−	−	+	+
Degradation of:						
Chondroitin sulfate	+	+	(+)	−	−	−
Tween 20	+	−	−	+	+	+
Tyrosine	+	−	(+)	+	+	+
Hemoglobin	+	−	+	+	+	+
Production of:						
Phenylalanine deaminase	(+)	−	−	−	+	+
Gelatinase	+	−	+	+	+	+
DNase	−	NG	−	−	−	+
Elastase	−	−	−	−	+	+
Cystine arylamidase	−	+	−	+	+	+
Trypsin	−	−	−	−	(+)	−
α-Chymotrypan	−	−	+	−	−	−
α-Galactosidase	+	−	+	−	−	+
β-Galactosidase	+	−	+	+	−	−
β-Glucoronidase	−	−	+	−	−	−
β-Glucosidase	+	+	−	+	−	−
α-Fucosidase	−	+	+	−	+	−
Brown pigment from tyrosine	−	−	−	−	−	+
Acetoin	−	+	+	−	−	+
Assimilation of:						
L-Arabinose	+	−	+	+	−	−
N-Acetyl-glucosamine	+	−	+	+	+	+
D-Maltose	+	−	+	+	+	+
Acid production from:						
D-Arabinose	−	−	+	−	−	−
L-Arabinose and D-xylose	+	−	(+)	+	−	−
D-Galactose	+	−	(+)	+	−	−
D-Glucose and D-mannose	+	(+)	(+)	+	(+)	−
D-Fructose	−	−	(+)	+	−	−
N-Acetyl-glucosamine	(+)	−	(+)	+	+	+
Amygdalin	(+)	−	(+)	+	+	+
Arbutin	−	−	−	+	−	+
Salicin	−	−	−	(+)	−	−
D-Cellobiose	+	+	+	+	−	−
D-Maltose	+	+	(+)	+	(+)	+
D-Lactose and D-melibiose	−	−	(+)	−	−	−
D-Sucrose	−	−	(+)	+	−	−
D-Trehalose	−	(+)	−	−	(+)	+
Inulin and D-raffinose	−	−	(+)	(+)	−	−
Glycogen	+	−	(+)	+	(+)	(+)
L-Fucose	−	−	(+)	−	−	−

Table 3. Antibiotic susceptibility results for 6 Michigan *Flavobacterium* spp. isolates as determined via the Kirby-Bauer disk diffusion method. R: resistant; S: sensitive. Number in parentheses is the mean diameter (in mm) of the zone of inhibition. PB: polymyxin-B (300 iu); SXT: trimethoprim-sulfamethoxazole (25 µg); P: penicillin G (10 iu); O129: 2,4-diamino,6,7-di-isopropyl pteridine (10 µg); FFC: florfenicol (30 µg); AMP: ampicillin (10 µg); E: erythromycin (15 µg); T: oxytetracycline (30 µg)

Isolate	SXT	PB	P	O129	FFC	AMP	E	T
T91	R (12.0)	R (9.5)	R (0)	R (0)	S (32.0)	R (11.5)	R (16.0)	S (23.0)
T75	R (11.0)	R (10.5)	R (0)	R (0)	S (33.0)	S (29.0)	S (31.0)	R (14.5)
T18	R (14.0)	R (9.5)	R (0)	R (0)	S (32.0)	S (13.0)	S (24.5)	S (30.0)
S87	R (14.0)	R (8.0)	S (13.0)	S (15.0)	S (25.0)	S (15.5)	R (17.5)	S (30.0)
S21	R (0)	R (11.5)	R (0)	R (0)	S (27.5)	R (0)	S (18.0)	S (27.0)
T76	S (18.0)	S (13.0)	R (0)	R (0)	S (24.0)	S (13.0)	S (24.5)	R (17.0)

Table 4. Bacterial challenge dose and percent mortality in 3 fish species challenged with 6 flavobacterial strains via intraperitoneal injection. Five fish belonging to each of the 3 salmonid species were used in each experimental challenge

Isolate	Chinook salmon		Brook trout		Brown trout	
	Dose (cfu)	Mortality (%)	Dose (cfu)	Mortality (%)	Dose (cfu)	Mortality (%)
T91	9.5×10^7	0	1.6×10^8	0	7.0×10^8	20
T75	3.8×10^8	0	8.5×10^7	0	2.0×10^8	0
T18	9.5×10^7	0	3.5×10^8	0	7.3×10^7	0
S87	2.3×10^8	0	2.0×10^8	0	3.4×10^8	0
S21	1.3×10^8	20	2.2×10^8	40	7.0×10^8	20
T76	1.3×10^8	0	1.4×10^8	0	1.5×10^8	40
Neg	0	0	0	0	0	0

from the liver to the body wall, swollen/enlarged spleens, and swollen/mottled/congested/edematous kidneys. T91-challenged fish also varied in the severity and range of pathological changes, which included gill pallor, bilateral exophthalmia, muscular ulceration and ecchymotic hemorrhage, hepatic swelling/congestion/enlargement, swelling of the spleen, swim bladder edema, and renal congestion and edema. In T75-infected fish, signs of disease included gill pallor, swollen, congested, hemorrhagic livers, swollen and enlarged spleens, and renal congestion, the presence of which varied within and amongst the 3 salmonid species. Pathological changes observed in fish challenged with T18 also varied, including gill pallor, pale and congested livers, swollen and enlarged spleens, and pale, swollen, congested, edematous kidneys. Lastly, in fish challenged with S87, observed signs of disease were variable and including gill pallor, congested and pale livers, swollen and enlarged spleens (Fig. 2C), multifocal ecchymotic hemorrhage in the swim bladder, swollen, congested, pale kidneys, and intracranial hemorrhage (Fig. 2D). In most instances, the aforementioned disease signs were most severe in fish that had died or were euthanized due to overt morbidity.

DISCUSSION

The results from this study not only illustrate the diversity of *Flavobacterium* spp. associated with diseased fishes in Michigan, but also support that a large proportion may represent novel flavobacterial taxa. For example, phylogenetic analysis demonstrated that the majority of Michigan *Flavobacterium* spp. isolates were phylogenetically distinct from all other described *Flavobacterium* spp., as evidenced by the formation of 17 distinct clusters according to both of the utilized phylogenetic methods. Furthermore, sequence analysis showed that the majority of the isolates within these clades were ≤98.7% similar to all described *Flavobacterium* spp. sequences, meaning they may represent new taxa within the genus (Bernardet & Bowman 2006). Additional genetic markers, such as *gyrB* or other housekeeping genes, were not used in this study because reference sequences for these loci are inconsistently available for *Flavobacterium* spp. and thus would not improve the resolution between the isolates of this study and those species that have already been described.

The presence of the newly described *F. plurextorum* in wild sea lamprey and hatchery-reared salmonids of Michigan (Table 1) is interesting, as this bacterium was recently isolated from the gills, livers, and eggs of farmed rainbow trout in Spain undergoing a septicemic disease outbreak (Zamora et al. 2013). Indeed, *F. plurextorum* isolates identified in this study (e.g. T164, T25, T165, T23, T96, and T95) were recovered from the gills of hatchery-reared fish suffering from disease outbreaks with elevated mortality, underscoring the potential pathogenicity of this bacterium. A similar observation was noted in

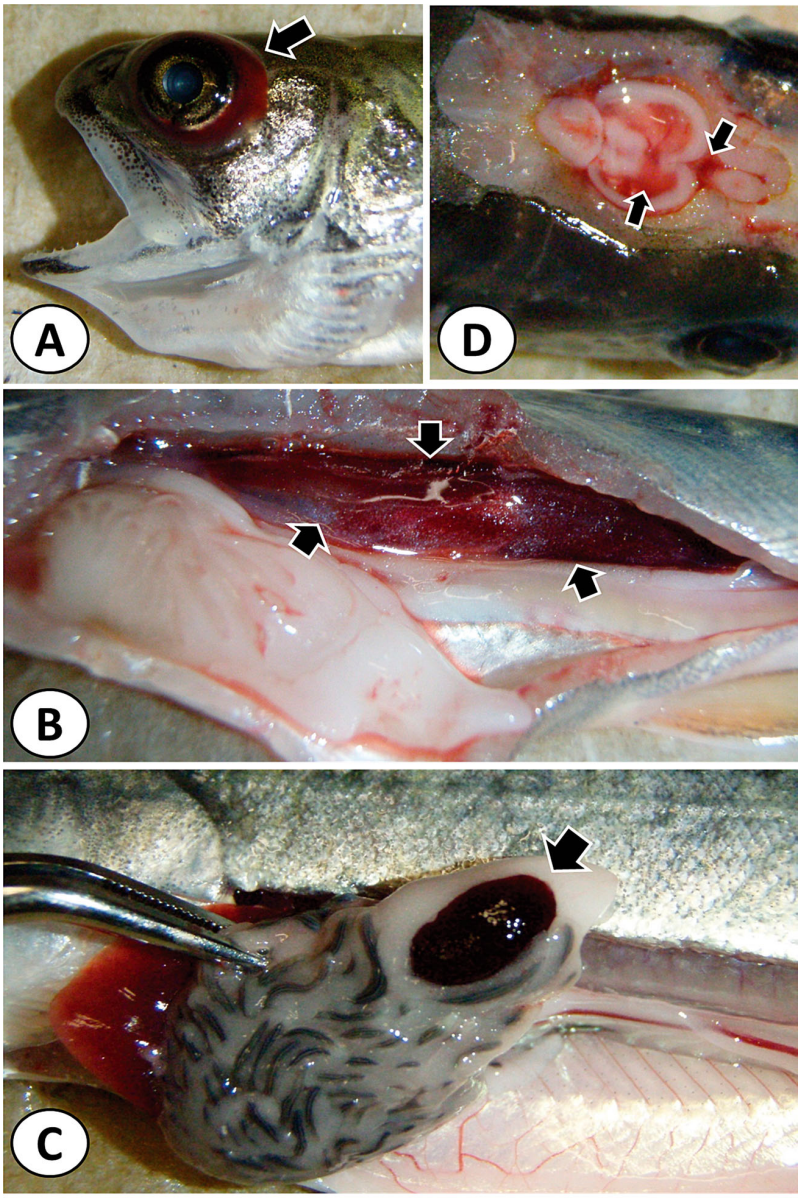

Fig. 2. Gross lesions observed in fish intraperitoneally injected with Michigan *Flavobacterium* spp. isolates. (A) Severe unilateral exophthalmia with diffuse periocular hemorrhage (arrow) in an S21-infected brook trout fingerling. (B) Severe diffuse hemorrhage present within the swim bladder (arrows), along with a small amount of red-tinged ascites within the body cavity, of an S21-infected brook trout fingerling. (C) Swollen spleen (arrow) in an S87-infected Chinook salmon fingerling. (D) Multifocal hemorrhage and edema (arrows) within the brain of an infected brook trout fingerling

wild, systemically infected fish with the recently described *F. spartansii* (e.g. T13).

Polyphasic characterization supported that T91, T75, S87, S21, and T76 are novel, as evidenced by their phenotypic and genetic distinctness when compared with their closest relatives (Table 1, Fig. 1; Tables S1, S2, S4–S6). However, additional experiments are needed to confirm the novelty of these flavobacteria. Another noteworthy finding of this study is the large number of Michigan *Flavobacterium* spp. isolates that were recovered from systemically infected fishes. While it is well known that some *Flavobacterium* spp., such as *F. psychrophilum*, *F. columnare*, and *F. branchiophilum*, negatively impact both wild and cultured fishes (Austin & Austin 2007, Bernardet & Bowman 2006, Shotts & Starliper 1999), the etiology of systemic disease caused by other *Flavobacterium* spp. is less well known. Only on rare occasions have other *Flavobacterium* spp. been implicated to cause fish diseases, and most often these outbreaks were associated with external lesions rather than systemic bacterial septicemia. For instance, *F. succinicans* (Anderson & Ordal 1961), *F. johnsoniae* (Christensen 1977, Carson et al. 1993, Rintamäki-Kinnunen et al. 1997), *F. hydatis* (Strohl & Tait 1978), and other uncharacterized flavobacteria (Borg 1960, Anderson & Conroy 1969, Lien 1988, Holliman et al. 1991) were recovered from the external lesions of diseased freshwater fish. However, reports of 'less well-known' and novel *Flavobacterium* spp. being recovered from the internal organs of fish in Africa (Flemming et al. 2007), Asia (Karatas et al. 2010, Suebsing & Kim 2012), Europe (Zamora et al. 2012, 2013, 2014), and South America (Kämpfer et al. 2012) are beginning to surface. Thus, the findings of this study, in conjunction with the findings of the aforementioned studies, illustrate that a much more diverse assemblage of flavobacteria are capable of systemically infecting fish. It is unknown whether the novel *Flavobacterium* spp. isolates described in this study are naturally present within the Great Lakes basin, or whether they were introduced into this region.

Experimental challenge studies with *Flavobacterium* sp. S21, which was originally recovered from a mortality event in hatchery-reared rainbow trout fingerlings, induced severe signs of disease in in-

fected fish. Gross pathological changes in naturally infected fish included enophthalmia, deep muscular ulceration, gill pallor, splenomegaly, and a swollen/pale/mottled liver (data not shown), while signs observed in experimentally infected fish included gross changes to the eyes, gills, spleen, and liver, as well as hemorrhaging within the brain and deterioration of the kidney. The bacterium was readily recoverable, in a pure form, from multiple internal organs of infected fish, indicating that a widespread infection involving multiple tissue systems (i.e. gastrointestinal, nervous, excretory, hematopoietic) had ensued. This bacterium was also recovered from the kidneys of all challenged fish, including those that survived until the end of the challenge period. Thus, these results provide evidence that *Flavobacterium* sp. S21 is likely a pathogen of salmonids.

It should be stated that experimental infections conducted via IP injection do not reproduce a natural infection route. Still, experimental challenge models using immersion, oral/anal intubation, and cohabitation routes of exposure for some of the well-known fish-pathogenic flavobacteria, such as *F. psychrophilum*, are rife with reproducibility problems despite being extensively studied (Holt 1987, Rangdale 1995, Decostere et al. 2000, Madetoja et al. 2000). In contrast, using an IP route of exposure produced reproducible results for *F. psychrophilum* challenges (Madsen & Dalsgaard 1999), while supplementing the experimental inoculum with horse serum and trace elements, as was done in this study, also reduced experimental variability (Michel et al. 1999). It is also noteworthy that, despite the circumvention of portions of the immune system during our IP infections, no mortalities occurred in any challenge involving *Flavobacterium* spp. isolates T75, T18, or S87. While it is possible that our experimental conditions may not have reproduced what is necessary for disease to ensue with these isolates, the fact that T75 and S87 were recovered from a portion of the challenged fish at the end of the study suggests that the mere presence of 10^8 cfu of flavobacteria within the body cavity of a fish does not ensure death of the host. In other words, despite bypassing portions of the innate immune system, a large dose of a non-pathogenic bacterium will not necessarily kill the host, further suggesting that isolates S21, along with isolates T76 and T91, may represent fish-pathogenic flavobacteria. It should be noted, however, that although the experimental challenge results of this study suggest pathogenicity for some of the aforementioned flavobacteria, the low numbers of challenged fish necessitates further investigation before any differences in pathogenicity can be fully determined. It is also notable that the host from which isolate S87 was recovered was co-infected with multiple motile *Aeromonas* spp, while isolates T18 and S21 were present in a mixed flavobacterial infection of the gills and external ulcers, respectively. However, isolates T75, T76, and T91 were recovered in pure culture and in the absence of other pathogens, including viral or parasitic pathogens.

Antibiotic susceptibility analysis showed that the 6 Michigan *Flavobacterium* spp. isolates were sensitive to florfenicol, an antibiotic currently approved by the United States Food and Drug Administration to treat disease outbreaks associated with *F. columnare* infections in farmed channel catfish and disease outbreaks associated with *F. psychrophilum* in aquacultured salmonids (www.fws.gov/fisheries/aadap/desk-reference_introduction.htm). Four of the 6 flavobacteria were also sensitive to oxytetracycline, which is approved to treat *F. columnare* outbreaks in freshwater-reared rainbow trout and *F. psychrophilum* outbreaks in freshwater-reared salmonids, while 5 of 6 isolates were resistant to trimethoprim-sulfamethoxazole, another antibiotic approved to treat some bacterial diseases of cultured fishes (www.fws.gov/fisheries/aadap/desk-reference_introduction.htm). In the event that disease outbreaks associated with any of these *Flavobacterium* spp. are associated with substantial losses in an aquaculture situation, it is imperative to have antibiotic sensitivity data.

In conclusion, the results of this study elucidate the heterogeneous assemblage of *Flavobacterium* spp. associated with diseased fish in Michigan. Clearly, further studies aimed at characterizing all of the flavobacterial strains highlighted in this study that likely comprise novel *Flavobacterium* spp., as well as studies further investigating what role(s) they may play as fish pathogens, commensals, or mutualists, are imperative if we are to understand host–flavobacteria–environment interactions in wild and cultured fishes.

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