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Polyphasic characterization reveals the presence of novel fish-associated *Chryseobacterium* spp. in the Great Lakes of North America

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ABSTRACT: Recent reports suggest an emergence of novel *Chryseobacterium* spp. associated with aquaculture-reared fish worldwide. Herein, we report on multiple *Chryseobacterium* spp. infecting Great Lakes fishes that are highly similar to previously detected isolates from Europe, Africa, and Asia but have never before been reported in North America. Polyphasic characterization, which included extensive physiological, morphological, and biochemical analyses, fatty acid profiling, and phylogenetic analyses based upon partial 16S rRNA gene sequences, highlighted the diversity of Great Lakes' fish-associated chryseobacteria and also suggested that at least 2 taxa represent potentially novel *Chryseobacterium* spp. Screening for the ability of representative chryseobacteria to elicit lesions in experimentally challenged fish showed that they induced varying degrees of pathology, some of which were severe and resulted in host death. Median lethal dose (LD_{50}) experiments for the isolate that elicited the most extensive pathology (*Chryseobacterium* sp. T28) demonstrated that the LD_{50} exceeded 4.5×10^8 cfu, thereby suggesting its role as a facultative fish-pathogenic bacterium. Histopathological changes in T28-infected fish included epithelial hyperplasia of the secondary lamellae and interlamellar space that resulted in secondary lamellar fusion, monocytic infiltrate, and mucus cell hyperplasia, all of which are consistent with branchitis, along with monocytic myositis, hemorrhage within the muscle, liver, adipose tissue, and ovaries, spongiosis of white matter of the brain, multifocal edema within the granular cell layer of the cerebellar cortex, and renal tubular degeneration and necrosis. The findings of this study underscore the widespread presence of chryseobacteria infecting Great Lakes fish.

KEY WORDS: *Chryseobacterium* · Fish disease · Great Lakes · Bacterial infections · *Flavobacterium*

INTRODUCTION

The genus *Chryseobacterium* (Family *Flavobacteriaceae*) contained 6 species when it was initially described (Vandamme et al. 1994), but with the advent of improved microbiological techniques and advanced molecular diagnostics, this genus now contains more than 80 described and proposed species (EzBioCloud; Kim et al. 2012). Along the way and in keeping with the taxonomic turmoil that has occurred within the family *Flavobacteriaceae*, the genus *Chryseobacterium* has been revised and restructured on multiple occasions (Kim et al. 2005, Kämpfer et al. 2009a,b). However, improved taxonomic clarity has been achieved through the widespread use of molecular techniques, including 16S rRNA gene sequencing, and the published standards for the description of novel chryseobacteria by the Subcommittee on the

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taxonomy of *Flavobacterium* and *Cytophaga*-like bacteria of the International Committee on Systematics of Prokaryotes (Bernardet et al. 2002).

Paralleling the expansion of the genus, an increasing number of *Chryseobacterium* spp. have recently been described that are associated with diseased farmed fish worldwide, including *C. piscicola* (Ilardi et al. 2009), *C. chaponense* (Kämpfer et al. 2011), *C. tructae* (Zamora et al. 2012b), *C. oncorhynchi* (Zamora et al. 2012a), and *C. viscerum* (Zamora et al. 2012c). During these outbreaks, external lesions (Kämpfer et al. 2011), deep ulcerations (Ilardi et al. 2010), and signs of septicemia (Zamora et al. 2012a,b) were reported in infected fish. Unfortunately, unraveling the pathogenesis of these novel fish-pathogenic chryseobacteria has received little attention. Concomitant with the apparent emergence of fish chryseobacteriosis, which is exacerbated by the bacterium's constitutive resistance to a wide spectrum of antibiotics that pose problems for chemotherapy (Bernardet et al. 2006), is an increased concern regarding the ability of *Chryseobacterium* spp. to cause rare, yet fatal, diseases in other animals, including humans (Bernardet et al. 2006).

In an effort to explore the ecology of fish-associated chryseobacteria and other flavobacteria in the Laurentian Great Lakes of North America, we recently performed phylogenetic analysis of several *Chryseobacterium* spp. recovered from diseased fishes. While a portion of the Great Lakes' isolates showed similarity to *C. viscerum, C. vrystaatense, C. chaponense, C. piscicola,* and *C. aquaticum* (Loch et al. 2013), the majority did not match previously described species, and some isolates were suspected of representing taxa that have never before been reported, such as *C. aahli* (Loch & Faisal 2014a). In this study, we further characterized the phylogeny of representative *Chryseobacterium* spp. isolates, which was followed by extensive phenotypic characterization and assessment of their ability to induce gross pathological lesions in experimentally infected fish, with the goal of expanding our understanding of the pathophysiology of chryseobacteriosis in fish.

MATERIALS AND METHODS

Fish sampling and bacterial isolates

The *Chryseobacterium* spp. isolates in this study were recovered from 9 fish species/strains that were cultured in hatcheries (n = 15) or collected from rivers and streams (n = 2; Table 1) in the watersheds of Lakes Michigan, Huron, and Superior. Among these, 11 isolates were recovered from 6 disease epizootics associated with mortality, while 6 were isolated from fish during health inspection or surveillance (Table 1). The fish species and organs from which bacteria were isolated are also presented in Table 1. Fish sampling and bacterial isolations were performed as previously described (Loch et al. 2013). Briefly, kidney tissues were aseptically collected using sterile disposable loops, inoculated directly onto plates of Hsu-Shotts medium (HSM; Bullock et

Table 1. *Chryseobacterium* spp. isolates that were analyzed using 16S rDNA sequencing and phylogenetic analyses, 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; M: isolate recovered during a mortality event; S: isolate recovered during health surveillance

Isolate ID	GenBank acc. no.	Closest relative (% 16S rDNA similarity)	Fish host species	Organ from which bacterium was recovered
T86	JX287899	*C. viscerum* (99.0%)	Muskellunge (H, M)	Kidney
T88	JX287901	*C. viscerum* (98.9%)	Muskellunge (H, M)	Gills
T87	JX287900	*C. viscerum* (99.0%)	Muskellunge (H, M)	Gills
T107	JX287902	*C. yeoncheonense* (99.1%)	Brown trout (H, M)	Fins
T130	JX287904	*C. aahli* (99.4%)	Brook trout (H, S)	Kidney
T24	JX287886	*C. piscium* (98.8%)	Steelhead (H, M)	Gills
T82	JX287895	*C. piscium* (98.7%)	Lake herring (H, M)	Kidney
T84	JX287897	*C. piscium* (98.7%)	Brown trout (H, M)	Gills
T83	JX287896	*C. piscium* (99.0%)	Lake herring (H, M)	Kidney
T31	JX287888	*C. piscium* (99.0%)	Steelhead (H, S)	Kidney
T115	JX287903	*C. chaponense* (99.6%)	Chinook salmon (W, S)	Kidney
T60	JX287890	*C. chaponense* (99.4%)	Rainbow trout (H, S)	Kidney
T63	JX287892	*C. piscicola* (99.6%)	Brown trout (H, S)	Fins
T39	JX287889	*C. viscerum* (99.2%)	Mottled sculpin (W, S)	Gills
T28	JX287887	*C. lactis* (98.8%)	Coho salmon (H, M)	Kidney
T72	JX287894	*C. piscium* (98.9%)	Lake herring (H, M)	Kidney
T85	JX287898	*C. aquaticum* (97.6%)	Brown trout (H, M)	Gills

al. 1986) supplemented with 4 mg l^{-1} neomycin sulfate, and incubated at 22°C for ≤7 d. In cases where disease signs were observed in the gills and fins, affected tissues were excised with sterile scissors and forceps and streaked directly onto HSM.

A total of 17 *Chryseobacterium* spp. isolates were maintained in Hsu-Shotts broth supplemented with 20% (v/v) glycerol at −80°C, and revived for analysis in this study.

16S rRNA gene amplification and phylogenetic analysis

For the 17 *Chryseobacterium* spp. isolates, DNA extraction, amplification of the 16S rRNA gene, and gene sequencing, as well as sequence comparisons and alignment to all formally described/candidate *Chryseobacterium* spp. were conducted as previously described (Loch & Faisal 2014b). Neighbor-joining analysis was performed using the Molecular Evolutionary Genetics Analysis software (MEGA; Ver. 5.0) and Bayesian analysis in MrBayes 3.2 (Ronquist et al. 2012) using the general time reversible (GTR) model with the previously described parameters (Loch & Faisal 2014b). However, 4 independent analyses were run, whereby the Markov chains (n = 12) were run for up to 100 million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of 0.05. In the event 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). Results from Bayesian analyses were visualized in FigTree v1.3.1 (Rambaut 2009).

Phenotypic characterization of representative *Chryseobacterium* spp. isolates

This extensive analysis was performed on 7 *Chryseobacterium* spp. isolates: T86, T28, T72, T83, T31, T24, and T115. These isolates were chosen as representatives of those associated with systemic infections in hatchery-reared fish with or without mortality events (T86, T28, T72, T83, T31, and T24), as well as systemic infections in wild fish (T115; Table 1). Assays for phenotypic characterization were selected as per the recommendations of the Subcommittee on the taxonomy of *Flavobacterium* and *Cytophaga*-like bacteria of the International Committee on Systematics of Prokaryotes for the description of novel bacterial taxa in general, and for members of the family *Flavobacteriaceae* in particular (Bernardet et al. 2002), and included the following.

Morphological, physiological, and biochemical characterization

Isolates cultured for 24 to 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, catalase (3% H_2O_2) and cytochrome oxidase (Pathotec test strips) activities, 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). Additional morphological, biochemical, and physiological characterization was performed as previously described (Loch & Faisal 2014b). 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. A list of the performed assays is displayed in Table 2.

Antibiotic susceptibility testing and fatty acid profiling

Chryseobacterium spp. isolates were tested for antibiotic susceptibility using the Kirby-Bauer disk diffusion method (Bauer et al. 1966). Cultures grown on HSM (24–48 h) were resuspended in sterile 0.85% saline, adjusted to an optical density (OD) of 0.5 at 600 nm in a WPA Biowave CO8000 Cell Density Meter, and 1 ml of the bacterial suspension was inoculated onto dilute Mueller-Hinton agar (Hawke & Thune 1992) without 5% calf serum in duplicate. Antibiotic-impregnated disks (Remel) 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 and cutoffs for sensitivity were as follows: polymyxin-B (300 iu), <8 mm = resistant, 8–12 mm = intermediate sensitivity, >12 mm = sensitive; oxytetracycline (30 µg), <15 mm = resistant, 15–18 mm = intermediate sensitivity, >18 mm = sensitive; trimethoprim-sulfamethoxazole (25 µg), <11 mm = resistant, 11–15 mm: intermediate sensitivity, >15 mm = sensitive; erythromycin (15 µg), <14 mm = resistant,

Table 2. Culture, biochemical, and physiological characteristics that exhibited variable results among the 7 *Chryseobacterium* spp. isolates examined in this study. +: positive test result; (+): weak positive test result; −: negative test result. API 20E, API 20NE, and API ZYM are commercially available identification galleries

Characteristic	Isolate						
	T86	T28	T72	T83	T31	T24	T115
Flexirubin-type pigment	+	+	+	+	+	+	−
Esculin hydrolysis	+	+	+	+	+	+	−
Hemoglobin lysis	+	+	+	+	+	+	−
Growth on:							
Marine agar	+	+	+	+	+	+	−
MacConkey agar	+	−	−	−	−	−	−
Cytophaga agar	+	+	+	+	+	+	+
Cetrimide agar	+	(+)	−	−	−	−	−
Growth at 2% NaCl	+	+	+	+	+	+	−
Growth at 3% NaCl	(+)	(+)	+	(+)	(+)	(+)	−
Esculin hydrolysis	+	+	+	+	+	+	−
Hemoglobin lysis	+	+	+	+	+	+	−
Production of:							
Indole	+	+	+	+	+	+	−
Phenylalanine deaminase	−	−	−	−	−	−	+
Gelatinase	+	+	+	+	+	+	−
Pectinase	+	+	−	−	−	−	+
Collagenase	+	−	−	−	−	−	−
DNase	+	+	(+)	+	+	+	−
Elastase	+	+	+	+	+	+	−
Degradation of:							
Chondroitin sulfate	+	+	−	−	−	−	−
Starch	+	+	−	−	−	−	−
Carboxymethyl cellulose	−	−	−	−	−	−	(+)
Tween 20	+	+	+	+	+	+	−
Tween 80	+	−	−	+	+	+	+
API 20E:							
β-galactosidase	−	+	−	−	−	−	−
Arginine dihydrolase	(+)	(+)	+	−	(+)	−	−
Tryptophane deaminase	+	+	+	+	+	+	−
API 20NE:							
Urease	+	+	+	−	−	+	−
Para-nitrophenyl-β-D-galactopyranosidase	−	+	−	−	−	−	−
API ZYM:							
Trypsin	+	−	−	+	+	−	−
α-chymotrypan	(+)	+	−	−	(+)	−	+
α-glucosidase	−	+	−	−	−	−	+
β-glucosidase	−	−	+	−	−	+	−
N-acetyl-β-glucosaminidase	+	+	+	+	+	+	−

14–18 mm = intermediate sensitivity, >18 mm = sensitive; ampicillin (10 µg), <12 mm = resistant, 12–13 mm = intermediate sensitivity, >13 mm = sensitive; florfenicol (30 µg; BD Diagnostics), <16 mm = resistant, 16–21 mm = intermediate sensitivity, >21 mm = sensitive; penicillin G (10 iu), <12 mm = resistant, >12 mm = sensitive; and the vibriostatic agent 0/129 (2,4-diamino,6,7-di-isopropyl pteridine; 10 µg), <7 mm = resistant, >7 mm = sensitive (Whitman 2004).

Fatty acid methyl ester analysis was performed as described by Sasser (1990) and Bernardet et al. (2005).

Experimental challenge studies

Experimental challenge experiments were performed in 2 stages. Initially we screened for the ability of the 7 chryseobacterial isolates to elicit gross lesions in experimentally infected fish. This was followed by quantitatively determining the median lethal dose (LD_{50}) of the isolate that induced the most severe pathological lesions in infected fish. All handling, maintenance, and experimental challenges 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*, brown trout *Salmo trutta*, and coho salmon *O. kisutch* were acclimated to laboratory conditions and held for a minimum of 8 wk prior to use in experimental challenges at the Michigan State University–University Research Containment Facility. 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 and optimal water parameters maintained throughout the experiments. In addition, ~1.5 yr old muskellunge *Esox masquinongy* maintained in our laboratory for >1 yr were maintained in the same fashion but were fed live fathead minnows *Pimephales promelas*. Prior to the experiment, subsets of each of the fish species, including fathead minnows, were euthanized via sodium bicarbonate-buffered tricaine methanesulfonate (MS-222, 250 mg l^{-1}; Argent Chemical Laboratories) and analyzed for the presence of bacteria capable of growing on trypticase soy agar at 22°C, flavobacteria (as described by Loch et al. 2013), *Renibacterium salmoninarum* (using a quantitative enzyme-linked immunosorbent assay, Faisal et al. 2012), parasites, and viruses that replicate in epithelioma papulosum cyprini (EPC) and Chinook salmon embryo (CHSE-214) cell lines.

Screening for the ability to cause lesions in fish infected via intraperitoneal (IP) injection

Chryseobacterial isolates used in experimental challenges were in their logarithmic growth phase, as determined in growth kinetic experiments that were performed prior to their use in experimental infections (Loch & Faisal 2014b). Initially, the ability of chryseobacterial isolates to induce pathological lesions in fish was tested in 5 fish species (5 fish species^{-1} isolate^{-1}). Chinook salmon (mean ± SD measurements: 5.8 ± 1.7 g; 8.6 ± 0.9 cm), brook trout (4.3 ± 1.4 g; 7.7 ± 0.9 cm), and brown trout (4.0 ± 1.3 g; 7.5 ± 0.7 cm) were anesthetized in carbonate-buffered MS-222 at a concentration of 100 mg l^{-1} and then IP injected with chryseobacteria suspended in phosphate-buffered saline (PBS; 100 µl fish^{-1}) containing 6.5 × 10^7 to 2.9 × 10^8 cfu. Control fish (n = 5) were inoculated with 100 µl of sterile PBS. In addition, *Chryseobacterium* spp. T28 and T86 were used to experimentally infect 5 fish from the species they were originally isolated from, namely coho salmon (6.8 ± 3.4 g; 8.9 ± 1.4 cm) for T28 and muskellunge (30.2 ± 6.0 g; 19.7 ± 1.0 cm) for T86. Groups of challenged and negative control fish were immediately placed in individual flow-through tanks (70 l). The flow rate was set at 1.26 l min^{-1}, and aeration was provided. Fish were monitored for 14 d, at which time survivors were euthanized. Fish were checked twice daily for morbidity/mortality and fed daily to apparent satiation. In the event that severe signs of morbidity were observed, the affected fish was euthanized using 250 mg l^{-1} of sodium bicarbonate-buffered 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 cytophaga agar (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.

Estimation of LD_{50} of *Chryseobacterium* T28 and histopathology

T28 was selected for use in further experiments to determine the LD_{50} of this bacterium. Four serial 10-fold dilutions of the T28 suspension (4.5 × 10^8 to 4.5 × 10^5 cfu in 100 µl) were IP injected into coho salmon (16.9 ± 4.7 g; 12.3 ± 1.3 cm; n = 10 fish dilution^{-1}), which is the original host fish species. Negative control fish (n = 10) were IP injected with 100 µl of sterile PBS. Challenged and control fish were monitored for 28 d, mortalities were immediately necropsied, and re-isolations were performed as described above. The LD_{50} was calculated using the method of Reed & Muench (1938).

Tissues of infected and control fish were fixed in phosphate-buffered 10% formalin, embedded within paraffin, sectioned at 5 µm, stained with hematoxylin and eosin (Prophet et al. 1992), and observed under a light microscope.

RESULTS

The 16S rRNA gene sequence analysis further confirmed that the 17 isolates are members of the genus *Chryseobacterium,* whereby they were most similar (97.6–99.6%) to 8 described species of *Chryseobacterium* (Table 1). Phylogenetic analysis using neighbor-joining and Bayesian analyses resulted in 14 of the 17 chryseobacteria forming 7 well supported clusters (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d113p113_supp.pdf), the majority of which were distinct from all described and candidate *Chryseobacterium* spp. For example, isolates T28 (posterior probability, PP = 100), T86, T87, and T88 (PP = 100, bootstrap value [BS] ≥70), T85 (PP = 85), T82 and T84 (PP = 91), and T24 and T72 (PP = 100) were robustly supported as being distinct from other described chryseobacteria (Fig. S1). Moreover, despite being highly similar (e.g. 99.4–99.6%) to *C. chaponense* according to 16S rRNA sequence analysis (Table 1), T115 and T60 were strongly supported as being phylogenetically distinct from the *C. chaponense* type strain (PP = 100, BS ≥70), as was the case for T107/T130 and the type strains of their closest relatives (*C. aahli* and *C. yeoncheonense*; PP = 100) and T63 and its closest relative (*C. piscicola*; PP = 100, BS ≥ 70). The topology of isolates T39, T31, and T83 could not be fully resolved according to either phylogenetic method (Fig. S1).

Phenotypic characterization analyses performed for *Chryseobacterium* spp. isolates T86, T28, T72, T83 and T31, T24, and T115 were as follows: when cultured on CA, the majority of the isolates produced colonies that were convex with entire margins, semi-translucent, and golden yellow in color that ranged in size from 1.0 to 2.5 (T86), 1.0 to 4.0 (T28, T83, and T31), and ~0.75 to 2.5 (T72) mm in diameter. Isolate T24 produced colonies that were convex with entire margins, opaque, and a pale yellow color that ranged

in size from 1.0 to 3.5 mm in diameter, while isolate T115 produced colonies that were semi-translucent, pale yellow in color, were convex with entire margins, and ranged in size from ~0.75 to 2.0 mm in diameter. The 7 *Chryseobacterium* isolates were non-motile, Gram-negative rods (1.0–3.0 µm in length) that did not contain cell wall-associated galactosamine glycans, and were able to grow on nutrient, trypticase soy, HSM and CA. All isolates grew at temperatures from 4 to 22°C (T86 and T28 grew weakly at 4°C) but not at ≥37°C. The isolates grew at salinities from 0 to 1% but not at 4 to 5%. None of the isolates produced acid from glucose or sucrose, and they produced an alkaline slant with no reaction in the butt on triple sugar iron (TSI). None of the isolates produced H_2S or were agarolytic, nor did they display alginase or chitinase activities. All isolates had catalase, cytochrome oxidase, and caseinase activities, were able to utilize citrate as a sole carbon source and produced a brown pigment in the presence of tyrosine. Additionally, none of the isolates produced acetoin, ornithine decarboxylase, lysine decarboxylase, reduced nitrate, or produced acid mannitol, inositol, sorbitol, rhamnose, melibiose, amygdalin, and arabinose on the API 20E, nor did they ferment glucose or assimilate D-glucose, L-arabinose, D-mannitol, D-mannose, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid on the API 20NE. On the API ZYM, all isolates were positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase (weak activity by T72), acid phosphatase, and Napthol-AS-BI-phosphohydrolase activities but were negative for lipase, α-galactosidase, β-galactosidase, β-glucoronidase, α-mannosidase, and α-fucosidase activities. On the API 50CH, none of the isolates produced acid from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, or potassium 5-ketogluconate. The biochemical and physiological assay results that were variable amongst the 7 isolates are depicted in Table 2. As displayed in Tables S1–S4 in the Supplement, the 7 chryseobacterial isolates were dissimilar from their 2 closest relatives (based on % 16S rRNA gene sequence similarity) in multiple phenotypic characteristics.

Antibiotic susceptibility tests demonstrated that isolates T86, T28, T72, T83, T31, and T24 were sensitive to trimethoprim-sulfamethoxazole and the vibriostatic agent O129 but resistant to penicillin, florfenicol, ampicillin, erythromycin, and oxytetracycline (Table 3). Sensitivity to polymyxin was variable amongst isolates (Table 3). Isolate T115 was unique in that it was resistant to trimethoprim-sulfamethoxazole and O129 but sensitive to penicillin. However, similar to the other isolates, it was resistant to florfenicol, erythromycin, oxytetracycline, and ampicillin (Table 3).

Fatty acid profiles of the 7 Michigan isolates revealed that *iso*-$C_{15:0}$, *iso*-$C_{17:1}$ ω9*c*, *iso*-$C_{17:0}$ 3-OH, and summed feature 3 ($C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*) were predominant, which is typical of the genus *Chryseobacterium* (Bernardet et al. 2006); smaller amounts of other fatty acids were also present (Table 4). Comparisons amongst the 7 chryseobacteria reaffirmed some of the phylogenetic relationships (Fig. S1). For example, T115 possessed the most dissimilar fatty acid profile (Table 4) and was also the most phylogenetically distinct from the other 6 characterized chryseobacteria. Similarly, isolate T83 was highly similar to isolates T24 and T31 (Table 4), which all fell into a robustly supported clade that also contained other chryseobacteria commonly associated with fish (Fig. S1). However T72, which also fell into the 'fish clade', was no more similar to the other 3 chryseobacteria (e.g. T83, T24, and T31) than it was

Table 3. Antibiotic susceptibility results for 7 *Chryseobacterium* spp. isolates as determined via the Kirby-Bauer disk diffusion method (number in parentheses is the diameter of the zone of inhibition in mm). S: sensitive; R: resistant; IS: intermediate sensitivity; SXT: trimethoprim-sulfamethoxazole (25 µg); PB: polymyxin-B (300 iu); 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
T86	S (25)	R (0)	R (0)	S (23.5)	R (0)	R (0)	R (0)	R (0)
T28	S (22)	R (0)	R (0)	S (21.5)	R (0)	R (0)	R (13)	R (11.5)
T72	S (16)	IS (9.5)	R (0)	S (25)	R (0)	R (0)	R (0)	R (0)
T83	S (27)	IS (9.5)	R (0)	S (29.5)	R (0)	R (9)	R (13.5)	R (14)
T31	S (27)	IS (8.5)	R (0)	S (30.5)	R (0)	R (9)	R (13)	R (13.5)
T24	S (24.5)	IS (8.5)	R (0)	S (34)	R (0)	R (0)	R (12)	R (12.5)
T115	R (0)	IS (12)	S (13)	R (0)	R (0)	R (0)	R (0)	R (0)

Table 4. Cellular fatty acid profiles (%) of 7 fish-associated *Chryseobacterium* spp. isolates using the commercial Sherlock Microbial Identification System (MIDI, version 4.0). Tr: trace amounts (<1%) detected; –: not detected

Fatty acid	Isolate						
	T86	T28	T72	T83	T31	T24	T115
iso-$C_{13:0}$	Tr	Tr	1.4	Tr	Tr	Tr	9.8
anteiso-$C_{13:0}$	–	–	–	–	–	–	4.8
iso-$C_{14:0}$	–	–	–	–	–	–	Tr
$C_{14:0}$	Tr	–	Tr	–	–	–	–
iso-$C_{15:1}$F	Tr	–	–	Tr	–	Tr	Tr
anteiso-$C_{15:1}$ A	–	–	–	–	–	–	Tr
iso-$C_{15:0}$	36.5	33.4	41.6	39.8	38.8	34.4	29.0
anteiso-$C_{15:0}$	Tr	Tr	3.0	1.2	1.2	2.7	23.9
iso-$C_{16:0}$	Tr	–	–	Tr	Tr	Tr	–
iso-$C_{16:1}$H	–	–	–	–	–	–	Tr
Summed feature 3[a]	11.1	10.7	9.8	6.4	7.5	7.5	4.2
$C_{16:1}$ ω5*c*	–	–	–	–	–	–	Tr
$C_{16:0}$	1.6	1.2	1.2	1.2	1.1	1.6	–
iso-$C_{15:0}$ 3-OH	2.9	3.2	3.0	3.5	3.5	3.0	2.4
$C_{15:0}$ 2-OH	–	–	Tr	Tr	Tr	Tr	2.1
iso-$C_{17:1}$ ω9*c*	17.6	23.7	18.5	21.8	22.5	23.4	8.7
anteiso-$C_{17:1}$ B/iso I	–	Tr	Tr	–	Tr	–	Tr
iso-$C_{17:0}$	2.0	1.5	Tr	1.5	1.0	1.5	Tr
iso-$C_{16:0}$ 3-OH	Tr	1.1	Tr	Tr	Tr	1.0	1.1
$C_{16:0}$ 3-OH	1.4	1.1	1.7	1.3	1.3	1.5	Tr
$C_{18:1}$ ω9*c*	Tr	Tr	Tr	Tr	Tr	Tr	Tr
iso-$C_{17:0}$ 3-OH	23.4	22.4	15.5	19.3	19.1	18.3	9.2
$C_{17:0}$ 2-OH	Tr	Tr	1.5	1.1	1.0	2.8	2.3

[a]As indicated by Del Carmen Montero-Calasanz et al. (2013), summed features are groups of 2 or 3 fatty acids that are treated together for the purpose of evaluation in the MIDI System and include both peaks with discrete equivalent chain-lengths (ECLs) as well as those where the ECLs are not reported separately. Summed feature 3 was listed as C16: ω6*c* and/or C16: ω7*c*

to T28, which fell well outside of this clade. When compared to the previously published fatty acid profiles, isolates T86, T83, T31, T24, and T72 were similar to their 2 closest relatives but also accompanied by some differences (Tables S1 & S2). Likewise, isolate T115 was similar to *C. chaponense* in many regards yet also distinct (e.g. a lower percentage of *iso*-$C_{16:0}$ 3-OH, 1.1 vs. 5.3% in *C. chaponense*; no *iso*-$C_{16:0}$ versus 3.2% in *C. chaponense*; Table S3). Isolate T28 was also similar to *C. lactis* and *C. nakagawai* in fatty acid profile, with the exception that T28 had nearly a 2.5 fold higher percentage of *iso*-$C_{17:1}$ ω9*c* (Table S4).

Growth kinetic experiments showed that the *Chryseobacterium* spp. isolates were in logarithmic to late logarithmic growth after 24 h of static incubation at 22°C. Thus, 18 to 24 h broth cultures were used for experimental infection. Analysis of a subset of fish prior to experimental challenges revealed the absence of any flavobacteria and other pathogens in their internal organs.

In the screening experiments, *Chryseobacterium* spp. isolates were recovered from the livers, spleens, kidneys, and brains of all fish that died prior to the end of the 14 d challenge period, with the exception of one T86-infected Chinook salmon that yielded growth from the liver, spleen, and kidney only. In all cases, bacteria recovered from experimentally challenged fish were identified as the original bacterial species utilized in the injection. The number of fish that died in each experimental challenge is listed in Table 5; no mortality occurred in any of the mock-challenged fish throughout the course of this study, nor was any bacterial isolate recovered from negative control fish.

The gross pathological lesions in fish infected with the 7 chryseobacterial isolates are listed in Table 5, whereby the most severe lesions were observed in fish infected with isolate T28. Some of the most pronounced lesions in *Chryseobacterium*-infected fish included exophthalmia and periocular hemorrhage (Fig. 1A), focal to multifocal gill hemorrhage and pallor (Fig. 1B), deep hemorrhagic ulceration of the muscle (Fig. 1C), intracranial hemorrhage (Fig. 1D), multifocal ulceration of the trunk and isthmus (Fig. 1E), ecchymotic hemorrhage within the lateral muscle (Fig. 1F), intracranial hemorrhage accompanied by hydrocephalus (Fig. 1G), and perinasal and intracranial hemorrhage (Fig. 1H). In multiple instances, the lesions in experimentally infected fish were similar to those observed in diseased fish from which the isolate was originally recovered (Table 5).

The pathogenicity of T28 was further confirmed by the LD_{50} experiments. Based upon our results, the LD_{50} for T28 was estimated to be $>4.5 \times 10^8$ cfu in ~17 g coho salmon. *Chryseobacterium* sp. T28 was recovered from the kidneys of all mortalities that occurred in the LD_{50} experiments, but no bacteria were recovered from any of the negative control fish.

Histopathological changes in T28-challenged coho salmon were assessed. Moribund and dead fish exhibited epithelial hyperplasia of the secondary lamellae and interlamellar space that resulted in secondary lamellar fusion (Fig. 2A,B), monocytic infiltrate and mucus cell hyperplasia within primary lamellae consistent with branchitis (Fig. 2C), monocytic myositis, hemorrhage within the muscle, liver, adipose tissue, and ovaries (Fig. 2D), pancreatitis, edema within the liver and interstitial tissue of the anterior kidney, renal tubular degeneration and necrosis (Fig. 2E), and

Table 5. Gross pathological lesions present in fish experimentally infected (intraperitoneally, IP) with 7 chryseobacterial isolates in this study, compared with lesions observed in naturally infected fish. Concurrent fish pathogens present in naturally infected fish are listed in the last column. CHS: Chinook salmon *Oncorhynchus tshawytscha*; BKT: brook trout *Salvelinus fontinalis*; MUE: muskellunge *Esox masquinongy*; BNT: brown trout *Salmo trutta*; COS: coho salmon *O. kisutch*

Isolate	Mortality in challenged fish	Lesions in experimentally infected IP challenged fish	Lesions in naturally infected fish	Concurrent pathogens (natural)
T86	4/5 (CHS), 2/5 (BKT), 1/5 (MUE), 1/5 (BNT)	Pale gills; flared opercula; multifocal muscular ulceration and hemorrhage; congested liver, enlarged spleen, pale, edematous, congested kidney; stomach containing a clear fluid; hemorrhagic enteritis; swim bladder, stomach, and intracranial hemorrhage	Dermal ulceration; fin erosion; swollen spleen; hepatic and renal pallor; visceral edema	*Flavobacterium columnare*
T28	3/5 (CHS), 3/5 (BKT), 2/5 (COS), 3/5 (BNT)	Unilateral exophthalmia; periocular hemorrhage; pale hemorrhagic gills; muscular ulceration; ascites; swollen enlarged spleen; edematous hemorrhagic kidney; distension of the stomach with a clear fluid; swim bladder, stomach, and intracranial hemorrhage	Corneal opacity and unilateral exophthalmia; hepatic congestion, swollen spleen; darkened mottled kidney; hemorrhagic enteritis	Motile *Aeromonas* sp.
T72	4/5 (CHS), 3/5 (BKT), 1/5 (BNT)	Bilateral exophthalmia; diffuse dermal hemorrhage and ulceration; pale hemorrhagic gills; hemorrhage of the body wall/muscle; congested edematous pale liver; swollen spleen; red-tinged ascites; pale edematous swollen kidney; fluid within stomach; swim bladder and intracranial hemorrhage	Dermal ulceration; diffuse dermal hemorrhage; fin base congestion; clear fluid swim bladder; pale liver; congested swollen friable edematous kidney; swim bladder and body wall hemorrhage	*Pseudomonas* sp.
T83	0/5 (CHS), 0/5 (BKT), 2/5 (BNT)	Bilateral exophthalmia; ocular and perioral hemorrhage; pale gills; congested swollen liver; swollen enlarged spleen; congested edematous kidney; hemorrhagic swim bladder	Petechial hemorrhage at base of fins; visceral edema; pale liver; fluid within the swim bladder lumen	*Flavobacterium* sp.
T31	2/4 (CHS), 1/5 (BKT), 0/5 (BNT)	Pale gills; unilateral exophthalmia; periocular hemorrhage; hemorrhage and ulceration at injection site; swollen liver; swollen enlarged spleen; hemorrhagic swim bladder; edematous hemorrhagic swollen kidney; peri-renal hemorrhage	None	None
T24	3/5 (CHS), 1/5 (BKT), 1/5 (BNT)	Unilateral/bilateral exophthalmia; perinasal, perioral, and intracranial hemorrhage; pale gills; deep muscular ulceration; diffuse hemorrhages on the ventrum; ascites; congested liver; swollen enlarged spleen; distension of the stomach with a clear fluid; swim bladder hemorrhage; hemorrhagic edematous kidney	None	Motile *Aeromonas* sp., *Flavobacterium* sp.
T115	0/5 (CHS, BKT, BNT)	Melanosis; swollen congested liver; swollen enlarged spleen; mottled kidney	Swollen friable spleen	None

splenic congestion. In coho salmon surviving until the end of the 28 d challenge period in the highest infectious dose, moderate to severe proliferative branchitis, pancreatitis, mild diffuse spongiosis in the white matter of the brain (Fig. 2F), focal edema in the liver, renal tubular degeneration and necrosis, and splenic congestion were evident. In the group challenged with the next lowest infectious dose, histological changes were similar but also included mild degeneration of the renal tubular epithelium, as well as hyperemia of the vessels and multifocal edema within the granular cell layer of the cerebellar cortex (Fig. 2G). In the groups challenged with the 2 lowest infectious doses, microscopic changes included mild proliferative branchitis, mild epicarditis, congestion and edema within the kidneys, and hepatic/splenic congestion. No major histological abnormalities were observed in the negative control fish.

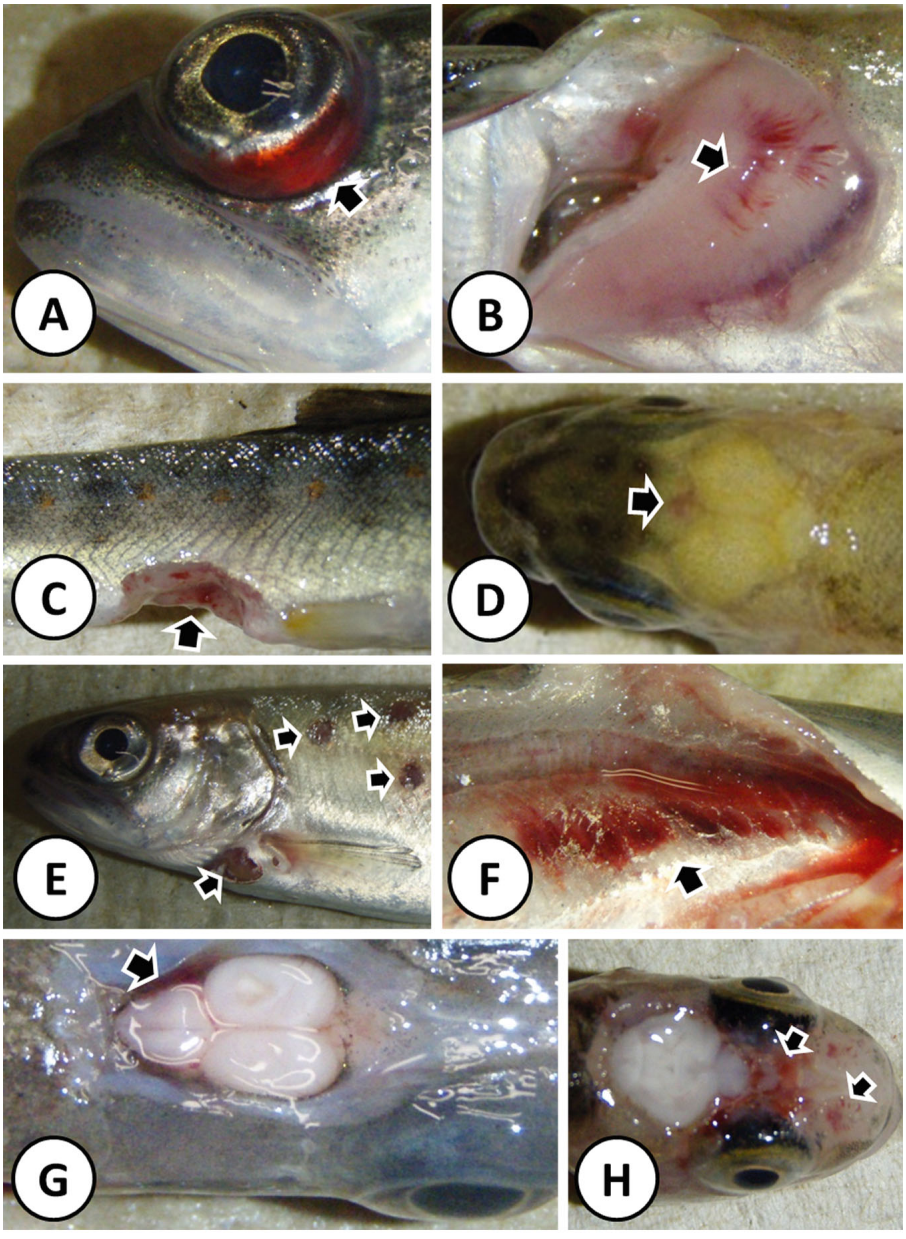

Fig. 1. Gross lesions observed in fish intraperitoneally injected with *Chryseobacterium* spp. isolates. (A) Unilateral exophthalmia and periocular hemorrhage (arrow) in a Chinook salmon *Oncorhynchus tshawytscha* fingerling infected with isolate T28. (B) Pallor and multifocal hemorrhage (arrow) within the gills of a T28-infected brown trout *Salmo trutta* fingerling. (C) A T28-infected brown trout fingerling with a deep hemorrhagic ulceration (arrow) in the ventral musculature. (D) Intracranial hemorrhage anterior to the optic lobes (arrow) in a T28-infected brown trout fingerling. (E) Multifocal ulceration (arrows) present on the trunk and isthmus of a Chinook salmon fingerling infected with isolate T72. (F) Ecchymotic hemorrhage (arrow) within the lateral muscle of a T86-infected Chinook salmon fingerling. (G) Intracranial hemorrhage (arrow) accompanied by hydrocephalus in a T86-infected muskellunge *Esox masquinongy* fingerling. (H) Intracranial and perinasal hemorrhage (arrows) in a T24-infected brown trout fingerling

DISCUSSION

Increasing reports of fish pathogenic *Chryseobacterium* spp. worldwide suggest that chryseobacteria are emerging as sources of disease outbreaks in farmed fishes. The findings from this study, in conjunction with our recent findings (Loch et al. 2013, Loch & Faisal 2014a) and those of Pridgeon et al. (2013), definitively demonstrate that a diversity of chryseobacteria are also associated with North Ame-

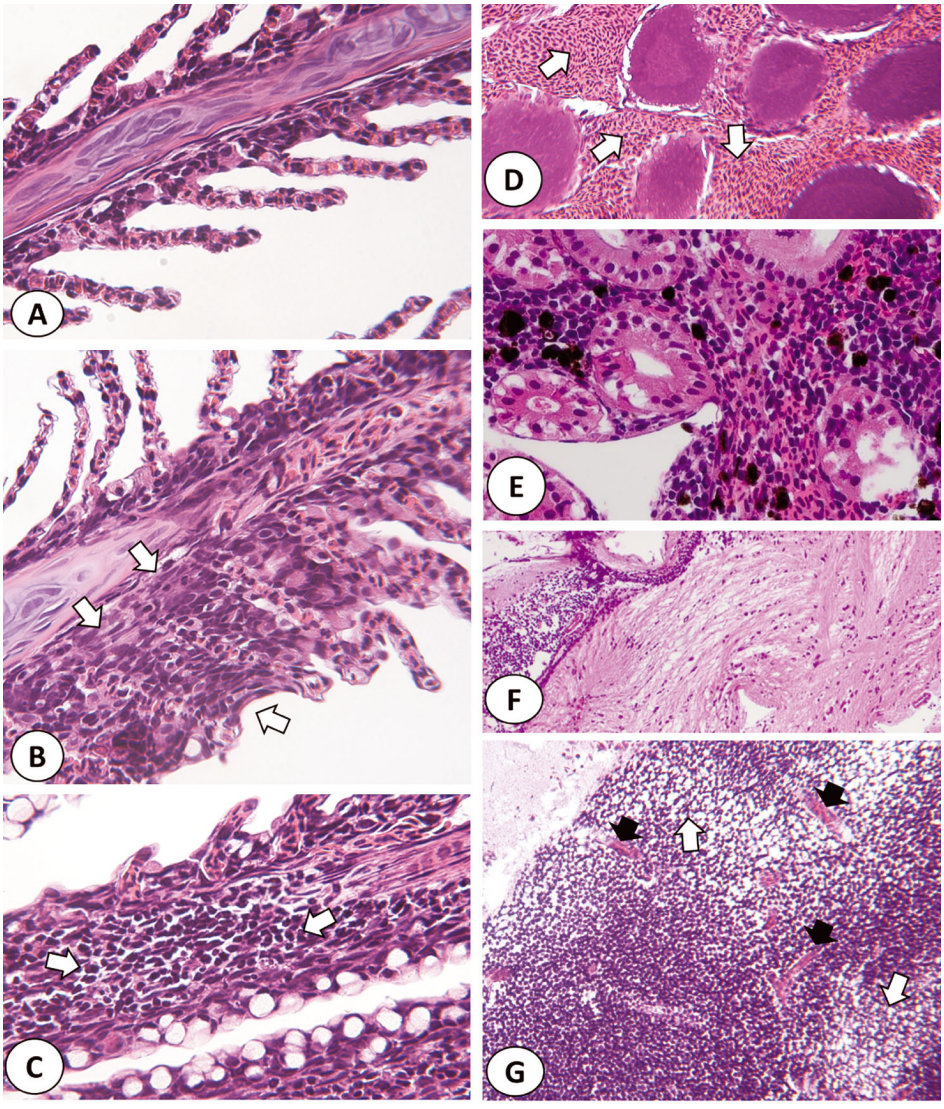

Fig. 2. Hematoxylin and eosin stained tissue sections from coho salmon *Oncorhynchus kisutch* (A) mock challenged and (B–F) challenged with *Chryseobacterium* sp. T28. (A) Gills showing normal secondary lamellae (400×). (B) Gills showing epithelial hyperplasia of the secondary lamellae and interlamellar space (arrows; 400×). (C) Primary lamellae with monocytic infiltrate (arrows) and mucus cell hyperplasia (400×). (D) Hemorrhage (arrows) within the ovaries (200×). (E) Renal tubular degeneration and necrosis (400×). (F) Mild diffuse spongiosis within the white matter of the brain (100×). (G) Granular cell layer of the cerebellar cortex showing hyperemia of the vessels (black arrows) and multifocal edema (white arrows; 100×)

rican fishes. Isolates highly similar to *C. piscicola, C. viscerum,* and *C. chaponense* were identified in Great Lakes fish stocks, each of which have been previously isolated from diseased salmonids in Chile and Finland (Ilardi et al. 2009, 2010), diseased rainbow trout *Oncorhynchus mykiss* in Spain (Zamora et al. 2012c), and diseased Atlantic salmon in Chile (Kämpfer et al. 2011). In all cases, the original isolations of these novel chryseobacteria were from farmed fishes (Ilardi et al. 2009, Kämpfer et al. 2011, Zamora et al. 2012c), possibly indicating that the stressors associated with aquaculture are necessary for chryseobacteriosis to ensue; however, this study shows that *C. chaponense*-like and *C. viscerum*-like bacteria are also associated with wild fishes.

Polyphasic characterization for 7 representative chryseobacterial isolates illustrated that they were all distinct, yet exhibited similarities, to described *Chryseobacterium* spp. For example, *Chryseobacterium* sp. T115 was highly similar to *C. chaponense* based upon genetic analysis, which was further supported using the polyphasic approach. However, some phys-

iological and biochemical differences existed between strain T115 and the *C. chaponense* type strain (Kämpfer et al. 2011), which possibly suggests strain divergence or that they are distinct species/subspecies. Although *C. chaponense* was originally recovered from diseased fish (Kämpfer et al. 2011), results from our experimental challenges demonstrated that strain T115 did not induce major pathological changes when administered IP to several salmonid species and did not result in mortality in any challenged fish. It is possible, however, that a different route of exposure (e.g. intramuscular injection, dermal abrasion followed by bath exposure, or immersion challenge) is a requisite for disease causation due to the pathophysiological processes that may follow.

Polyphasic characterization also highlighted the distinctness of isolates T72, T83, T31, and T24. According to sequence analysis of the 16S rRNA gene, *Chryseobacterium* sp. T72, T83, T31, and T24 were 98.8 to 99.0% similar to *C. piscium*, which was first isolated from fish caught from the South Atlantic Ocean in South Africa (De Beer et al. 2006). However, 98.7 to 99.0% has been suggested to be a threshold above which further analyses should be carried out to delineate bacterial species (Stackebrandt & Ebers 2006). Phylogenetic analyses, phenotypic characterization, and fatty acid profiling collectively suggested that these 4 chryseobacterial isolates are closely related to *C. piscium*, although some biochemical and physiological discrepancies were noted. As such, we suspect that T83/T31 may represent unusual strains of *C. piscium*, as do T72/T24. All 4 of these isolates also fell into a robustly supported clade that contains chryseobacteria that are well known for their associations with fish and/or fish disease (Fig. S1), including *C. balustinum* (Bernardet et al. 2006), *C. scophthalmum* (Mudarris & Austin 1989, Mudarris et al. 1994), and *C. piscium* (De Beer et al. 2006).

Interestingly, *C. piscium* was originally considered to be a spoilage organism (De Beer et al. 2006); however, the 4 *C. piscium*-like isolates in this study were originally recovered from diseased lake herring (*Coregonus artedi*; T83 and T72), diseased steelhead (*O. mykiss*; T24), and systemically infected steelhead (T31) being raised in Michigan hatcheries. Indeed, the 4 isolates elicited pronounced gross pathological changes in fish challenged via IP injection, which is significant because a portion of the naturally infected fish also harbored other bacterial pathogens, thus preventing the lesions from being solely attributed to the chryseobacteria. Thus, the recovery of *C. piscium*-like bacteria from multiple disease events in Michigan hatcheries, in conjunction with the findings of this study, suggests that they are potentially pathogenic to salmonids.

Results from polyphasic characterization suggested that the 2 remaining isolates may represent novel *Chryseobacterium* spp. For example, the identity of T86 was ambiguous according to its % 16S rDNA similarity, falling within the 98.7 to 99.0% threshold of Stackebrandt & Ebers (2006) to help ascertain whether a bacterium is a novel species, while neighbor-joining and Bayesian phylogenetic analyses supported T86 as distinct from all other chryseobacteria, including *C. viscerum*. Indeed, T86 was phenotypically distinct from *C. viscerum* in a number of enzymatic, biochemical, and fatty acid characters. Experimental challenge studies with *Chryseobacterium* sp. T86 by IP injection also demonstrated that this bacterium was capable of solely inducing significant gross pathology in infected fish, including the original host, the muskellunge.

T28 was distinct from its closest relatives phylogenetically, physiologically, biochemically, and in cellular fatty acid profile (e.g. *iso*-$C_{17:1}$ ω9*c*). Thus, we were unable to ascribe isolate T28 to a defined *Chryseobacterium* sp. and suggest that it represents a novel bacterial species pending DNA–DNA hybridization experiments. Pilot experimental challenge studies with T28 in 4 different salmonid species, including coho salmon, its original host, confirmed this bacterium's ability to generate severe lesions which were frequently accompanied by death. Moreover, fish infected with this bacterium showed the most severe gross signs of disease in all of the chryseobacteria examined in this study. Indeed, *in vitro* experiments demonstrated the ability of this bacterium to proteolyze a number of substrates that are constituents of the host extracellular matrix (Alberts et al. 2002), including gelatin, elastin, and chondroitin sulfate (Table 2). Experiments to enumerate the LD_{50} of this bacterium via IP challenge showed that it exceeded 4.5×10^8 cfu in 17 g coho salmon, which is within the range for a facultative pathogen. It is important to note that the challenge model used in this study does not mimic a natural infection, in that the external defenses of the fish were bypassed. However, experimental infections via more natural routes of exposure (e.g. immersion) with some fish-pathogenic members of the family *Flavobacteriaceae* were, until very recently, notoriously difficult to induce (Long et al. 2014), whereas IP challenges had proven to be reproducible (Madsen & Dalsgaard 1999). Nevertheless, histopathological changes were

induced in fish that were challenged with 10^4 cfu, thereby indicating that isolate T28 is capable of negatively impacting the health of an infected host without necessarily killing it. Indeed, histological evaluation of turbot experimentally challenged with *C. scophthalmum* by Mudarris & Austin (1992) also revealed damage to the kidneys in the form of degeneration and necrosis of the renal tubular epithelial cells, which was observed in our study as well. Mudarris & Austin (1992) also observed epithelial hyperplasia of the secondary lamellae and proliferation of the interlamellar cells, both of which were also observed in this study and are indicative of a proliferative branchitis. In light of the extensive gross and microscopic changes observed in the gills of fish challenged with *Chryseobacterium* sp. T28, further experiments using a more natural immersion route of exposure are warranted in order to further investigate the pathogenesis of this bacterium and its possible role in diseases affecting the gills of fish.

The 7 fish-associated *Chryseobacterium* spp. isolates recovered from Michigan fishes exhibited a striking resistance to the vast majority of the antibiotics that were tested in this study, which has also been reported in other chryseobacteria recovered from aquatic animals (Michel et al. 2005). These findings mirror the problems associated with treatment of chryseobacterial infections in humans (Kirby et al. 2004, Chou et al. 2011) and are troubling in light of the fact that the Great Lakes chryseobacteria are resistant to 3 of the 4 major antimicrobials approved for use in aquaculture by the US Food and Drug Administration (www.fws.gov/fisheries/aadap/desk-reference_introduction.htm). It is important to note, however, that such resistance is a hallmark of this genus and thus does not necessarily implicate anthropogenic activities as the cause but rather is an indication that insufficient research to develop and approve an efficacious antibiotic has yet to be undertaken. Thus, finding an efficacious chemotherapeutant to treat chryseobacteriosis outbreaks in fish should be studied further. Likewise, determining whether *Chryseobacterium*-infected fish/fomites can serve as a source for chryseobacterial infections in humans is imperative, especially because their presence seems to be much more widespread than previously recognized.

In conclusion, findings from this study provide evidence that a diversity of fish-associated chryseobacteria, some of which are likely novel species, are singly capable of inducing pathological changes in experimentally challenged fish. Clearly, further studies elucidating the epizootiology and pathogenesis of the diverse assemblage of fish-pathogenic *Chryseobacterium* spp. are needed, which are ongoing in our laboratory.

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