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REVIEW

Emerging flavobacterial infections in fish: A review

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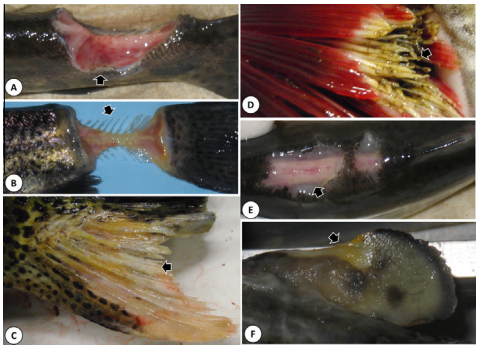
GRAPHICAL ABSTRACT



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ABSTRACT

Flavobacterial diseases in fish are caused by multiple bacterial species within the family Flavobacteriaceae and are responsible for devastating losses in wild and farmed fish stocks around the world. In addition to directly imposing negative economic and ecological effects, flavobacterial disease outbreaks are also notoriously difficult to prevent and control despite nearly 100 years of scientific research. The emergence of recent reports linking previously uncharacterized flavobacteria to systemic infections and mortality events in fish stocks of Europe, South America, Asia, Africa, and North America is also of major concern and has highlighted some of the difficulties surrounding the diagnosis and chemotherapeutic treat-

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ment of flavobacterial fish diseases. Herein, we provide a review of the literature that focuses on *Flavobacterium* and *Chryseobacterium* spp. and emphasizes those associated with fish.

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Introduction

Flavobacterial diseases were first reported by Davis in 1922 and have since been recognized as a serious threat to wild and propagated fish stocks alike. Originally, these diseases were attributed to three bacteria within the family Flavobacteriaceae [1]; namely, *Flavobacterium psychrophilum*, the etiological agent of bacterial cold water disease and rainbow trout fry syndrome [2,3]; *Flavobacterium columnare*, the causative agent of columnaris disease [4,5]; and *Flavobacterium branchiophilum*, the putative agent of bacterial gill disease [5,6]. Others have reported additional *Flavobacterium* spp. associated with diseased fish, including *Flavobacterium johnsoniae* [7], *Flavobacterium succinicans* [8], *Flavobacterium hydatis* [9], as well as other uncharacterized yellow-pigmented bacteria (Austin and Austin [10] and references therein). In acute flavobacteriosis, cumulative mortality upwards of 70% can occur among affected fish stocks, while survivors may suffer poor growth and spinal abnormalities (reviewed in Austin and Austin [10]). In subacute and chronic infections, flavobacteriosis elicits lingering mortalities that can lead to continuous economic losses [11]. Taxonomy and speciation of this family has undergone many revisions; therefore, throughout this review, the most currently recognized terminology of genera and species will be used.

With the recent advances in molecular biology and biotechnology, several novel genera within the family Flavobacteriaceae (e.g., *Chryseobacterium*, *Elizabethkingia*, *Tenacibaculum*, and *Ornithobacterium*) have emerged that encompass pathogens of fish, amphibians, reptiles, birds, and mammals, including humans [1]. Among these, *Tenacibaculum* spp. are important pathogens of marine fishes that have been reviewed elsewhere [12,13] and will not be discussed further herein. Rather, this review predominantly focuses on the genera *Flavobacterium* and *Chryseobacterium*, within which multiple novel species have been described over the last decade in association with diseased fishes from around the world [14–26].

History of flavobacterial diseases in fish

In the process of studying protozoan parasites at the U.S. Fisheries biological station in Fairport, Iowa, Davis [27] observed multiple fish mortality events during the summers of 1917–1919 that he associated with an unidentified bacterium. The affected fish, which included buffalofish (*Ictiobus bubalus*), sunfish (*Leopomis* spp.), common carp (*Cyprinus carpio*), largemouth and smallmouth bass (*Micropterus salmoides* and *Micropterus dolomieu*), crappie (*Pomixis* spp.), warmouth (*Leopomis gulcosus*), yellow perch (*Perca flavescens*), white bass (*Morone chrysops*), brook trout (*Salvelinus fontinalis*), bluntnose minnow (*Pimephalus notatus*), channel catfish (*Ictalurus punctatus*), and bullhead catfish (*Ameiurus* spp.), were cultured in aquaria and earthen ponds. Davis [27] noted that affected fish displayed "dirty-white or yellowish areas" on the body, whereby lesions developed and caused death within 24–72 h. Fins (especially the caudal fin) were eroded and, in more severe cases, only "mere stubs" remained. There was also necrosis of the gills visible as white patches that spread rapidly, causing death. The author also observed mortalities in wild fishes of the Mississippi River associated with this bacterium. Although he was unable to isolate the bacterium in any of these outbreaks, he observed large numbers of long, slender, flexible rods associated with the necrotic lesions of the skin and gills of affected fish that formed "column-like masses"; thus, he named the bacterium *Bacillus columnaris*. Two decades later, Ordal and Rucker [28] successfully isolated the yellow-pigmented bacterium and named it *Chondrococcus columnaris* due to its association with cartilage and what they characterized as the production of fruiting bodies and microcysts. However, Garnjobst [29] showed that *C. columnaris* did not produce such structures and reclassified the bacterium within the genus *Cytophaga*, as *Cytophaga columnaris*. While agreement on the negative impacts this bacterium had on fish stocks was well accepted, as evidenced by its inclusion in the list of notifiable fish diseases outlined in the British Diseases of Fish Act of 1937 [10], the disagreement on its taxonomy continued when it was provisionally placed into the genus *Flexibacter* [30,31]. Extensive molecular and phylogenetic studies by Bernardet et al. [32] placed the bacterium in the genus *Flavobacterium* as *F. columnare*, where it has remained to the present day and is recognized as the etiologic agent of columnaris disease.

Another yellow-pigmented bacterium also has a long history in association with freshwater fish diseases. In 1926 and 1927, Davis [33,34] reported multiple disease outbreaks in fingerling brook trout and steelhead (*Oncorhynchus mykiss*) reared in Vermont and New York, which he attributed to an unknown bacterium that was associated with damage to the gills. He noted slow chronic mortalities that increased with time when temperatures began to rise. Davis [34] noted that the bacteria formed "luxuriant growth over the surface of the gills" that coincided with increased mucus production, clubbing of the gill lamellae, and proliferation of gill epithelium causing fusion of the secondary lamellae. Otherwise, fish appeared normal until death. Other researchers observed similar disease outbreaks and isolated yellow pigmented bacteria from affected fish, but they were unable to reproduce the disease experimentally [35–37]. Wakabayashi [38] successfully recovered a yellow pigmented bacterium from hatchery-reared salmonids from Japan and Oregon that was distinct from those used in the aforementioned studies of Rucker and Bullock and successfully reproduced the disease. This bacterium was classified as *Flavobacterium branchiophila* [6], which became *F. branchiophilum* [32]. It is now widely believed that *F. branchiophilum* is a causative agent of bacterial gill disease (BGD; [39]); however, environmental parameters and other bacteria are also believed to play a role in some outbreaks of BGD.

A third unidentified yellow-pigmented bacterium was associated with serious disease in rainbow trout (*O. mykiss*) fingerlings reared at the national fish hatchery in Leetown, West Virginia [40]. Although Davis was unable to isolate this bacterium, he observed huge numbers of non-motile bacterial rods in scrapings taken from deep ulcerations present on the caudal peduncle of affected fish. These bacteria did not form the characteristic "columns" associated with *F. columnare*. Soon thereafter, Borg [41] reported a similar pathological condition among diseased hatchery-reared juvenile coho salmon (*O. kisutch*) from Washington. In this case, a bacterium was successfully isolated from the kidneys and external lesions of systemically infected fish. Borg [41,42] reproduced the disease in experimentally challenged fish, with signs that included ulcerations at the caudal peduncle that went so deep so as to almost detach the tail from the body. While this bacterium was initially placed in the order Myxobacterales and named *Cytophaga psychrophila* [42,43], it was reclassified as *Flexibacter psychrophilus* [30] and later as *Flavobacterium psychrophilum* [32]. As its name implies, *F. psychrophilum* grows best at low temperatures (~15 °C) and frequently causes disease when water temperatures are below 10 °C. In North America, the terms low temperature disease [42] and bacterial cold water disease [44] are used to describe outbreaks associated with this bacterium, whereas outbreaks in Europe are commonly referred to as rainbow trout fry syndrome [45,46].

As is quite clear from the aforementioned history of fish-pathogenic flavobacteria, their taxonomy has been quite tumultuous. While the fish health literature is replete with reports of fish diseases associated with unidentified or partially characterized yellow-pigmented bacteria referred to as *Cytophaga* spp. and *Cytophaga*-like bacteria, *Flexibacter* spp., *Flavobacterium*-like spp., and "myxobacteria" e.g., [10,47–53], many now belong to the family Flavobacteriaceae.

The family Flavobacteriaceae

The family Flavobacteriaceae (Phylum Bacteroidetes; Class Flavobacteriia; Order Flavobacteriales; [54]) was first suggested by Jooste [55]. Despite being mentioned by Reichenbach [56], the family was not validated until 1992 by Reichenbach and its formal description published by Bernardet et al. [32]. Included within the family Flavobacteriaceae at that time was the type genus, *Flavobacterium* [32], along with the genera *Chryseobacterium* [57,58], *Bergeyella* [58,59], *Empedobacter* [58,60], *Capnocytophaga* [61,62], *Ornithobacterium* [58], *Weeksella* [63], and *Riemerella* [64,65], along with the species that would eventually comprise the genera *Myroides* [66,67], and *Tenacibaculum* [32,68]. Bernardet et al. [69] published minimal standards for describing new taxa within the family Flavobacteriaceae, which in addition to the genera mentioned above also included *Coenonia* [70], *Psychroserpens* and *Gelidibacter* [71], *Polaribacter* [72], *Psychroflexus* [73], *Salegentibacter* [74], *Cellulophaga* [75], and *Zobellia* [76], along with two generically misclassified taxa [69]. In subsequent years, the family has rapidly expanded from <20 genera to >100 described and candidate genera [77,78].

Characteristics of this family according to Bernardet [77] are as follows: Nonspore-forming short to long filamentous rods that stain Gram negative, are motile via gliding or non-motile, and are non-flagellated with rare exceptions (i.e., *Polaribacter irgensii*) [72]. Colony shape can vary from round and convex to rhizoid and flat, and can strongly adhere to the surface of the agar (i.e., *F. columnare*). Although some colonies do not pigment, most contain a non-diffusible yellowish to orange pigment due to the presence of carotenoid and/or flexirubin. Growth is typically aerobic, but some genera exhibit micro-aerophillic to anaerobic growth. Nitrates are not typically reduced, whereas oxidase and catalase activities are common. Most genera contain species that degrade organic substrates, including proteins (e.g., casein, gelatin, etc.), simple and complex carbohydrates (e.g., starch, esculin, pectin, chitin, carboxymethylcellulose), and lipids (e.g., Tween). Many species are halophilic and mesophilic, while others are halotolerant and psychrophilic. Branched saturated, branched monounsaturated, and branched hydroxy C_{15} to C_{17} fatty acids are frequently present in large amounts. The term flavobacteria will be used throughout this literature review to encompass members of the family Flavobacteriaceae.

Flavobacteria commonly reside in extremely diverse habitats, ranging from fresh and marine aquatic environments, soils, foods, beverages and their processing plants, as well as human and veterinary hospitals (reviewed in Bernardet and Nakagawa [1]). Many flavobacteria are pathogenic to a multitude of organisms, including plants [79], invertebrates [80], amphibians [81], reptiles [82], birds [64], and mammals [83], including humans [84]. Because the research presented herein focused on the genera *Flavobacterium* and *Chryseobacterium*, the remainder of this literature review will be comprised of information pertaining to them specifically.

The genus Flavobacterium

The genus *Flavobacterium* was described in the first edition of Bergey's Manual of Determinative Bacteriology [85] in 1923,

at which time it consisted of 46 species that included Gram negative, Gram positive, Gram variable, and flagellated bacteria [86]. By the publication of the eighth edition of Bergey's Manual in 1974, the majority of the flagellated and Gram positive strains were systematically removed, leaving only 12 species within the genus [86]. This taxonomic upheaval continued, whereby only 7 species remained in 1984 [87]; then, the extensive work published by Bernardet et al. [32] in 1996 resulted in the type species, *F. aquatile* [88], being the only original member to be retained within the genus *Flavobacterium*. Other species assimilated into the genus *Flavobacterium* at that time included *F. psychrophilum, F. branchiophilum, F. columnare, F. hydatis, F. succinicans, F. johnsoniae, F. flevense, F. pectinovorum,* and *F. saccharophilum* [32]. With much of the taxonomy settled, minimal standards for describing novel flavobacteria were published [69] in 2002, at which point the number of *Flavobacterium* spp. was 15. Over the next 4 years, 11 more *Flavobacterium* spp. were described [79], bringing the total to 26, and the number of formally described *Flavobacterium* spp. has rapidly expanded to over 100 published and proposed species at the time this review was written [http://www.ezbiocloud.net/eztaxon; 78].

Members of the genus *Flavobacterium* are Gram negative rods that range from 0.3 to 0.5 μm in diameter and from 1.0 to 40.0 μm in length. All species are non-motile or display gliding motility (the presence of flagella has not been reported). It should be noted that the degree by which flavobacteria move by gliding is optimal in media with low nutrient and high moisture contents. Colonies contain non-diffusible, non-fluorescent, flexirubin and/or carotenoid – type pigments, giving them a pale to bright yellow appearance. Optimal growth occurs from 20 to 30 °C for most species, though growth is better at 15–20 °C for the more psychrophilic species. The majority of *Flavobacterium* spp. grow on nutrient and trypticase soy agars (TSA) without the need for growth factors, though the dominant fish pathogenic species (i.e., *F. psychrophilum, F. columnare, F. branchiophilum*) are not able to grow on TSA. Some species are able to oxidize carbohydrates, but strong proteolytic activity is almost universally present. The predominant fatty acids present within members of the genus are *iso*-$C_{15:0}$, $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*, $C_{15:0}$, *iso*-$C_{17:0}$ 3-OH, *iso*-$C_{15:0}$ 3-OH, $C_{15:1}$ ω6*c*, *iso*-$C_{16:0}$ 3-OH, *iso*-$C_{15:1}$ G, *iso*-$C_{15:0}$ 2-OH, and *anteiso*-$C_{15:0}$. The type species is *F. aquatile* [88].

Flavobacterium spp. vary in the ease with which they are cultured on microbiological media. For example, many of the species that inhabit soil and freshwater grow readily on agar plates made from commercially available media (e.g., nutrient agar and TSA), as is also the case for the marine species (e.g., marine agar; reviewed in Bernardet and Bowman [79]). However, a portion of the freshwater fish-pathogenic species (see *Flavobacterium* spp. as pathogens below) is fastidious and requires specialized culture media, such as cytophaga agar [89], Shieh's medium [90], Hsu-Shotts medium [91], tryptone yeast extract salts medium (TYES; [44]), and medium # 2 [92], to name a few. Some of these media and their derivatives were made more selective by incorporation of polymyxin-B, neomycin, tobramycin, and/or vancomycin to avoid overgrowth by less fastidious bacteria that may also be present in an inoculum, especially from external lesions of fish. Optimal incubation temperatures vary widely and depend upon the species, but most of the fish-pathogenic species grow best from 15 to 25 °C [79].

The morphological, physiological, and biochemical characteristics that help to differentiate *Flavobacterium* from other genera within the family Flavobacteriaceae were extensively reviewed by Bernardet [77], and differential characters among *Flavobacterium* spp. are presented in Bernardet and Bowman [54]. In addition to bacterial culture and subsequent identification via phenotypic tests [10], many other means for detection and identification were developed. Whole-cell agglutination [43,93], fluorescent antibody tests (FAT; [94,95]), enzyme-linked immunosorbent assays [95,96], *in situ* hybridization [97], loop-mediated isothermal amplification (LAMP; [98,99]), polymerase chain reaction (PCR; [100–106]), immunomagnetic separation in conjunction with flow cytometry [107], quantitative PCR [108,109], and DNA array-based multiplex assay [110] are used to detect and identify *F. psychrophilum, F. columnare,* and/or *F. branchiophilum*. It is noteworthy, however, that few diagnostic reagents specific for the lesser-known fish associated flavobacteria exist, which makes their identification more difficult and laborious.

Ecology of Flavobacterium spp.

Although usually considered psychrophilic or psychrotolerant, some flavobacteria are also able to grow at temperatures of 37 °C (e.g., *F. granuli, F. columnare, F. suncheonse,* and *F. succinicans*), or even 40–45 °C (e.g., *F. defluvi, F. indicum, F. croceum*) [54]. However, the vast majority are recovered from cool, cold, or even polar habitats (reviewed in Bernardet and Bowman [54]). *Flavobacterium* spp. reside in diverse habitats, including freshwater streams, lakes, and sediments (e.g., [111,112]), deep wells [57], glaciers and arctic ice (e.g., [113,114]), plants and plant material (e.g., [115,116]), soils (e.g., [117]), freshwater shrimp ponds [118], marine sediments (e.g., [119]), seawater (e.g., [120]), wastewater treatment systems (e.g., [121,122]), on marine algae (e.g., [123]), and even within air-conditioning units [79].

Flavobacterium spp. have also been detected intracellularly in amoebae [124] and from the guts of earthworms (*Aporrectodea caliginosa*; [125]), butterflies [126], mosquitos [127], nematodes [128], leeches [129], and in association with corals (e.g., [130]), marine sponges [131], and marine mammals, such as beaked whales (*Ziphius cavirostris*; [132]). Caution should be exercised when referring to the primary literature documenting *Flavobacterium* spp. from many other animals, including humans, because many taxa originally belonging to the genus *Flavobacterium* were subsequently moved to other genera (e.g. *Flavobacterium breve,* now *Empedobacter brevis*, [58]; *Flavobacterium meningosepticum*, now *Elizabethkingia meningoseptica*, [133]). By far and away, the most plentiful reports of *Flavobacterium* spp. in associated with animals are those that document their presence on fish and fish products. For example, *Flavobacterium* spp. have been detected within the intestines [134,135], on the gills [136], fins [8], in the mucus [79], from eggs [137] and reproductive fluids [138], and from internal organs of cool, cold, and warm-water fishes (reviewed in Shotts and Starliper [5], Bernardet and Bowman [79], and Austin and Austin [10]). Although some *Flavobacterium* spp. are commensal [43,139,140], a number are major pathogens of freshwater and marine fishes.

Flavobacterium spp. as pathogens

A few reports have documented *Flavobacterium* spp., such as *F. johnsoniae*, with diseases in plants [79,141,142]. Flavobacterial infections were also occasionally reported in amphibians [27,137,143], and occasionally the bacteria were associated with disease in humans. For instance, an outbreak of respiratory disease in a group of workers from a textile plant occurred in the 1980s and was attributed to a *Flavobacterium* sp. strain that proliferated within an air-conditioning unit in the facility [79,144,145]. Most recently, *F. lindanitolerans* was recovered from the ascites of a child in China that died of fatal pulmonary edema and hemorrhage [146].

Flavobacterium spp. as fish pathogens

Since the initial report of Davis [27], multiple members of the genus *Flavobacterium* have been recognized as serious fish pathogens worldwide; namely, *F. columnare,* [27,28,91]; *F. psychrophilum* [3,40,45,46,147,148]; and *F. branchiophilum* [6,38,39]. Extensive reviews have been written on fish-pathogenic *Flavobacterium* spp. (e.g., [5,10,79]), which highlight the predominance of *F. psychrophilum, F. columnare,* and *F. branchiophilum* in fish health literature. The three aforementioned reviews described the impacts that the "big three" fish-pathogenic *Flavobacterium* spp. have on fish stocks worldwide and additional publications provided even greater depth concerning the impacts of *F. psychrophilum* (e.g., [2,3,44,148,149]), *F. columnare* (e.g., [91,150–152]), and *F. branchiophilum* (e.g., [6,39,153–156]). Thus, while the importance of *F. psychrophilum, F. columnare,* and *F. branchiophilum* as etiologic agents of fish disease is undeniable, the remainder of the review focuses on the "less well-known" freshwater fish-associated flavobacteria.

"Other" Flavobacterium spp. associated with diseased fishes

F. johnsoniae is occasionally associated with disease in fish. Christensen [7] first mentioned *F. johnsoniae* in association with diseased fish, followed by the report of Carson et al. [157] of *F. johnsoniae* causing shallow ulcerative lesions on the skins, fins, and jaws of juvenile aquacultured barramundi (*Lates calcarifer*) in Australia. In an attempt to fulfill Koch's postulates, Soltani et al. [158] utilized an isolate of *F. johnsoniae* from the disease outbreak in barramundi and challenged various fishes via bath exposure. Juvenile barramundi were the only studied species that were susceptible to infection with *F. johnsoniae*, and infections ensued only after fish were stressed thermally [158]. Rintamäki-Kinnunen et al. [159] also found an *F. johnsoniae*-like bacterium associated with external lesions on the gills, jaws, skin, and fins in multiple aquacultured salmonids in Finland. Most recently, *F. johnsoniae* and *F. johnsoniae*-like isolates were associated with disease in aquacultured longfin eels (*Anguilla mossambica*), rainbow trout, and koi (*C. carpio*) in South Africa [15], in cultured Russian sturgeon (*Acipenser gueldenstaedtii*) in Turkey [160], and in farmed rainbow trout in Korea [161].

Another *Flavobacterium* sp. implicated as a putative agent of fish disease is *F. hydatis* (formerly *Cytophaga aquatilis*; [9,32]). This bacterium was first isolated from the gills of propagated salmonids displaying "signs of bacterial gill disease" in Michigan by Strohl and Tait [9], but its pathogenicity was not assessed. Others have also recovered a similar bacterium from the gills of fish suffering from bacterial gill disease epizootics [10,79], thus suggesting a role as an opportunistic fish pathogen. Similarly, *F. succinicans* was originally isolated from the water and eroded caudal fin of a Chinook salmon (*Oncorhynchus tshawytscha*) fingerling at the University of Washington School of Fisheries (Seattle, Washington) and from a "lesion" on an adult Chinook salmon collected from the Brownlee Dam on the Snake River in Hells Canyon, Idaho [8]. However, the original author suggested that this bacterium was not a fish pathogen but rather a commensal, and further studies were not conducted.

Most recently, a number of novel *Flavobacterium* spp. were isolated from diseased fish in South America, Europe, and North America. *Flavobacterium chilense* was originally recovered from an external lesion of a farmed rainbow trout in a mixed culture with *F. psychrophilum*, while *F. araucananum* was recovered from the kidneys and external lesions of farmed Atlantic salmon (*Salmo salar*) in a mixed culture with *F. psychrophilum* [18], both of which were cultured in Chile. The original isolations of *F. oncorhynchi* took place in Spain, whereby the bacterium was recovered from the livers and gills of farmed rainbow trout with disease signs that were suggestive of an *F. psychrophilum* infection [19]. Likewise, *F. plurextorum* was originally recovered from the livers and eggs of farmed rainbow trout in Spain that were suffering from a septicemia [22], while *F. tructae* and *F. piscis* were recovered from the liver and gills and kidney and gills, respectively, of farmed diseased rainbow trout in Spain [23]. Three proposed *Flavobacterium* spp. (e.g., *F. collinsii, F. branchiarum,* and *F. branchiicola*) were also recovered from farmed rainbow trout in Spain in 2008 and 2008, though no signs of disease were reported [162]. In North America, *F. spartansii* was recovered from the kidneys of feral spawning Chinook salmon, as well as from the gills of hatchery-reared Chinook salmon, in the Great Lakes basin [26].

In addition to the aforementioned fish-associated *Flavobacterium* spp., other partially characterized and/or unidentified *Flavobacterium* spp. have periodically been reported from diseased fishes. Indeed, in their review, Shotts and Starliper [5] make brief mention of "other poorly defined *Flavobacterium*-like organisms" that have been implicated as facultative fish pathogens, while a similar statement that implicates partially characterized yellow-pigmented bacteria as agents of fish disease is made by Austin and Austin [10]. For example, Holliman et al. [163] documented a disease outbreak in captive-reared rainbow trout (*O. mykiss*) supplied with unfiltered lake water in Windermere, England, that was attributed to an unidentified "*Cytophaga*-like" bacterium, which generated a 16% mortality over 10-days and mortality abated after administration of oxytetracycline. On cytophaga agar, nearly pure bacterial growth was cultured from external lesions. Holliman et al. [163] found that they possessed the characters of "*Cytophaga*-like bacteria" that were distinct from *F. aquatile, F. psychrophilum, F. columnare* and indicated that this bacterium had never been previously reported in association with fish disease. Additionally, the authors confirmed pathogenicity via immersion, intraperitoneal, and intramuscular routes of transmission in rainbow trout and Atlantic salmon.

Similarly, Bowman and Nowak [164] detected a *Flavobacterium* sp. from the gills of net-penned Atlantic salmon in Tasmania that concurrently suffered from amoebic gill disease. Their isolate was most similar to *F. frigidarium* according to 16S rDNA sequences. It was not clear,

however, what role this bacterium had played in the disease process. Indeed, a number of unidentified "*Cytophaga*-like bacteria" were also reported in association with disease outbreaks in marine fishes (e.g., [50–52]).

The genus Chryseobacterium

The genus *Chryseobacterium* was originally created by Vandamme et al. [58] for six bacterial taxa that, at that time, were classified as members of the genus *Flavobacterium; F. balustinum, F. indologenes, F. gleum, F. meningosepticum, F. indoltheticum,* and *F. scophthalmum*. In keeping with the taxonomic upheaval that many of the taxa within the family Flavobacteriaceae endured, an additional genus, *Elizabethkingia*, was subsequently created for two species within the genus *Chryseobacterium*; namely, *C. meningosepticum* and *C. miricola* [133]. Since then, more taxonomic clarity has been achieved through the widespread use of improved molecular techniques and guidelines for description of novel *Chryseobacterium* species [69]. By 2006, the genus *Chryseobacterium* had expanded to 10 species, along with one unvalidated species (e.g., *C. proteolyticum*; [1]), but the genus contained >60 species when this review was written.

Members of the genus *Chryseobacterium* are non-motile, Gram negative, straight rods that are usually 1–3 μm in length and ~0.5 μm in width. None of the species within this genus have flagella, nor do they display gliding motility or swarming growth. Colonies range from pale to a bright golden yellow color due to the presence of a non-diffusible flexirubin-type pigment. *Chryseobacterium* spp. grow well on commercial media (i.e. TSA, brain heart infusion, marine, blood, nutrient, and Mueller Hinton agars [reviewed in 77,165]) at 4–42 °C (ideal incubation temperature of 20–30 °C for most species [165]) and at salinities of up to 5%, depending upon the species. Catalase and oxidase activities are present, isolates are strongly proteolytic, and most are resistant to numerous antibiotics. The predominant fatty acids contained by members of this genus are *iso*-$C_{15:0}$, *iso*-$C_{17:1}$ ω9*c*, *iso*-$C_{17:0}$ 3-OH, and *iso*-$C_{15:0}$ 2-OH/$C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*. The type species is *Chryseobacterium gleum* [Holmes et al. [87]].

The phenotypic and biochemical characteristics that differentiate *Chryseobacterium* spp. from other closely related members of the family Flavobacteriaceae were reviewed by Bernardet [77], while differential characters among the various *Chryseobacterium* spp. were presented in Bernardet et al. [166]. Presumptive identification of a *Chryseobacterium* sp. is often based upon phenotypic characters (e.g., Gram negative, non-motile rods that produce bright yellow colonies due to the presence of flexirubin-type pigments; possess oxidase and catalase activities; produce a *Chryseobacterium* spp. profile on commercial galleries; [14]), after which a definitive identification is based upon polyphasic characterization, including biochemical, morphological, and physiological characterization, fatty acid profiling, and sequence/phylogenetic analyses [69]. Additional techniques that have most recently been utilized to identify *Chryseobacterium* spp. include matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS; [167]) and PCR amplification of the 16S and internal transcribed spacer (ITS) rDNA and subsequent sequence analysis [168].

Ecology of Chryseobacterium spp.

Akin to the genus *Flavobacterium*, members of the genus *Chryseobacterium* inhabit diverse habitats. For example, *Chryseobacterium* spp. were recovered from soils (e.g., [169–171]), plant roots (e.g., [172]), flowers [173], decaying plant material [174], and maple sap [175]. Interestingly, some plant-associated *Chryseobacterium* strains inhibited plant-pathogenic fungi [172]. *Chryseobacterium* spp. were also recovered from freshwater creeks [176], lakes [177], their sediments [178], water cooling systems [179], drinking water [180], lactic acid beverages [181], beer bottling plants [182], bioreactor sludge [183], polluted soil [184], marine sediment [185], and permafrost [186]. In contrast to *Flavobacterium* spp., multiple studies that examined the bacterial assemblages of glaciers and Antarctic ice have not detected *Chryseobacterium* spp. ([165] and references therein).

Chryseobacterium spp. are associated with a multitude of animals. For instance, they have been detected from the midgut of mosquitos (*Culicoides sonorensis*, [127]; *Culex quinquefasciatus,* [187]), within cockroach guts (*Periplaneta americana*, [188]), millipede feces (*Arthrosphaera magna*, [189]), and penguin guano (*Pygoscelis adeliae*, [190]), gut homogenates of freshwater copepods (*Eudiaptomus gracilis*, [191]), bird feathers [192], cow's milk (e.g., [193–196]), and from raw meats and chicken [197–199]. *Chryseobacterium* spp. were recovered from the mucus of apparently healthy fish [165,200] and thus, they may be commensalistic. However, *Chryseobacterium balustinum* was first isolated from the scales of halibut (*Hippoglossus hippoglossus*) freshly harvested from the Pacific Ocean [201] where it was considered as a spoilage organism [10]. In this context, Engelbrecht et al. [202] recovered multiple *Chryseobacterium* spp. from marine fish caught in South Africa that produced H_2S and proteolyzed multiple substrates (e.g., casein, gelatin). Engelbrecht et al. [202] reported pungent and stale odors in muscle extracts, suggesting involvement in fish spoilage. Similarly, *C. piscium*, which was first isolated from fish in the south Atlantic Ocean of South Africa [203], was suggested to be a spoilage organism due to the presence of urease and phenylalanine deaminase activities. However, González et al. [204] suggested that *Chryseobacterium* spp. were not an important cause of spoilage in fish, because they comprised less than 1% of the bacterial communities of the fish that they sampled.

Chryseobacterium spp. as pathogens

As was mentioned for *Flavobacterium* spp., caution should be exercised when referring to the literature regarding *Chryseobacterium* spp. as pathogens, because *Chryseobacterium meningosepticum*, which caused numerous infections, was reclassified to the genus *Elizabethkingia* [133]. Nevertheless, *Chryseobacterium* spp. were recovered from multiple diseased organisms. For example, *C. indologenes* is pathogenic to the soft tick (*Ornithodoros moubata*, [205]), whereas other *Chryseobacterium* spp. were recovered from diseased turtles [82] and frogs [206,207]. In addition, multiple *Chryseobacterium* spp. were recovered from human clinical sources [57,208–211]. For example, *Chryseobacterium indologenes* has caused bacteremia in humans on numerous occasions [212–214] with multiple clinical manifestations, such as hospital-acquired pneumonia, peritonitis, surgical wound infections [212], and cellulitis [215]. *Chryseobacterium* spp. were also

isolated from burn wounds [216], eye infections [168], and pneumonia in newborns [217]. Such infections are nosocomial in nature and frequently associated with indwelling devices (e.g., catheters, tracheal tubes, ventilators; [165]).

Chryseobacterium infections in fish

The first isolation of *Chryseobacterium balustinum* was reported in 1929 from the scales of halibut (*Hippoglossus hippoglossus*) freshly harvested from the Pacific Ocean [201]. However, these isolates are no longer available in culture collections [165], and the current type strain was isolated from the heart blood of a freshwater dace (*Leuciscus leuciscus*) that had signs of septicemia [218] as reported in 1959. When this isolate was experimentally injected into multiple fish species, it caused mortality [218], thus suggesting its pathogenic potential [165].

Mudarris and Austin [219] reported a disease outbreak in farmed turbot (*Scophthalmus maximus*) that occurred in Scotland in 1987 associated with a novel *Chryseobacterium* sp. that they later named *C. scophthalmum* [58,220]. They recovered "dense pure cultures" of this bacterium from the gills and viscera of fish that exhibited hyperplasia of the gills, hemorrhage of the eyes, skin, and jaw, necrosis and hemorrhage of the brain, stomach, intestine, liver, and kidney, and ascites within the peritoneum [219]. They also recovered the same bacterium from apparently healthy adult and juvenile wild turbot. The same authors then experimentally assessed the pathogenicity of *C. scophthalmum* in juvenile turbot in seawater and rainbow trout in freshwater using both immersion exposure and intraperitoneal injection and found similar disease signs as what was seen in naturally infected fish. The authors recovered the same bacterium from infected fish, and fulfilled Koch's postulates. Moreover, injection of bacterial cell homogenates resulted in the same disease signs, but injections of either lipopolysaccharide (LPS) or cell supernatant did not produce any lesions [219]. The same authors later examined the histopathological changes associated with the natural and experimental *C. scophthalmum* infections [221] and reported swelling, necrosis, and edema within the gills, as well as epithelial hyperplasia of the secondary lamellae and proliferation of the interlamellar cells that "filled up the spaces between the secondary lamellae." Degeneration and necrosis of the renal tubular epithelial cells, dilatation and necrosis of the glomeruli, necrosis of interstitial renal tissue, focal necrosis of hepatic cells, edema of the bile ducts, and necrosis of the mucosal

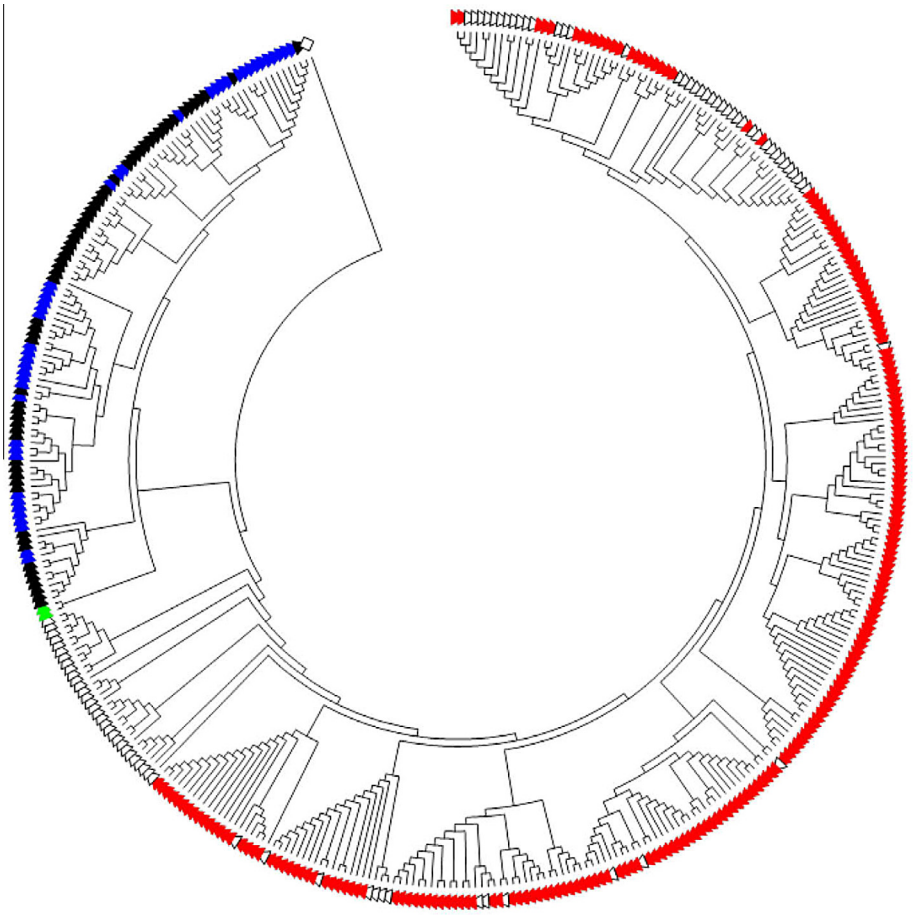

Fig. 1 Dendrogram based upon partial 16S rRNA gene sequence analysis, which was generated using the neighbor-joining method in Molecular Evolutionary Genetics Analysis (MEGA) version 5, depicting the phylogenetic relationships between Great Lakes fish-associated *Flavobacterium* (red triangles) and *Chryseobacterium* (blue triangles) spp. and described and candidate *Flavobacterium* (white triangles) and *Chryseobacterium* (black triangles) spp. The closely related *Elizabethkingia meningoseptica* and *E. miricola* (green triangles) were also included. The tree was rooted with *Capnocytophaga ochracea* U41350 (white diamond).

epithelial cells of the intestine were also noted. Pathological changes were not observed in either the heart or spleen.

More recently, Bernardet et al. [14] performed polyphasic characterization on 52 *Chryseobacterium* spp. isolates recovered from diseased fish in Belgium, Finland, France, Spain, Taiwan, Singapore, and Cambodia. While the majority of these isolates could not be ascribed to a definitive species, 14 clusters were defined and 3 of these were believed to represent novel *Chryseobacterium* spp. [14]. In addition, two of the isolates recovered from external lesions of farmed Atlantic salmon in Finland were identified as *C. joostei*, which was originally isolated from raw cow's milk in South Africa [193]; however, experiments investigating the pathogenicity of these strains were not reported. The authors did suggest that many of the *Chryseobacterium* spp. they studied represented facultative fish pathogens, because many were recovered from imported fish that were stressed [14].

Most recently, a number of novel *Chryseobacterium* spp. were recovered from fish, some of which were diseased. For example, *C. piscicola* was isolated from diseased Atlantic salmon and rainbow trout in Chile [16], diseased Atlantic salmon in Finland [222], and was later suggested to be moderately virulent in experimental challenges [222]. Likewise, *C. chaponense* was recovered from the fins, gills, and external lesions of diseased Atlantic salmon being farmed in Chile, in mixed culture with *F. psychrophilum* [17]. *Chryseobacterium viscerum* and *C. oncorhynchi* were originally isolated from the livers and gills of diseased rainbow trout (*O. mykiss*) in Spain [20,21]. In addition, *Chryseobacterium aahli* was described from the kidneys of systemically-infected, hatchery-reared lake trout (*Salvelinus namaycush*) and from the necrotic fins of hatchery-reared brown trout (*Salmo trutta*) of the Great Lakes of North America [25].

Interestingly, a number of *Chryseobacterium* spp. that are more frequently associated with human infections or human consumables were recently recovered from fish. For example, *C. hominis*, so named for its penchant for causing infections in humans [209], was isolated from the kidneys of a pufferfish (*Arothron hispidus*) [223,224] in 2005. In addition, *C. shigense*, which was originally isolated from a beverage in Japan [181], was recovered from farmed rainbow trout fry undergoing multiple disease outbreaks in Spain [225] in 2008 and 2009. In 2012, *C. indologenes*, which is increasingly associated with human infections [212–215], was identified as a cause of disease and mortality in farmed yellow perch (*P. flavescens*) in the United States and confirmed to be pathogenic to perch via experimental challenge [226].

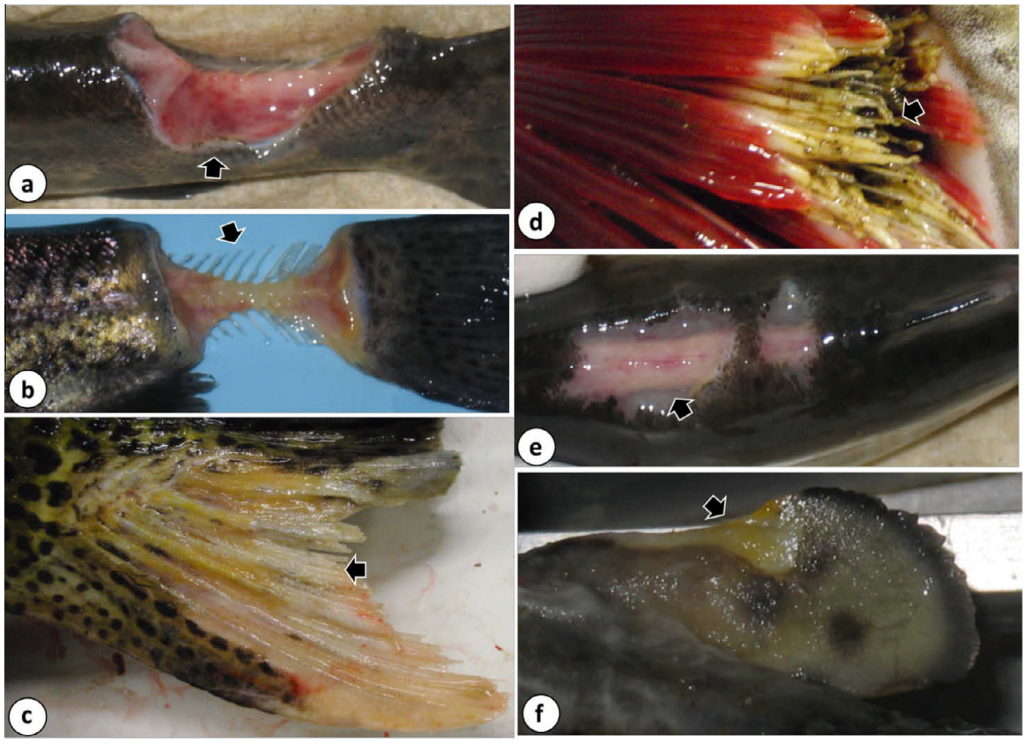

Fig. 2 Gross pathology in Great Lakes fishes infected with flavobacteria. (a) Deep muscular ulceration and concurrent hemorrhage (arrow) of the caudal peduncle in a rainbow trout infected with *F. psychrophilum*, (b) *Flavobacterium psychrophilum*-associated ulceration of the caudal peduncle that has left the vertebral column (arrow) fully exposed. Note also the yellowish discoloration of the lesion due to mats of the yellow-pigmented bacterium, (c) extensive erosion and necrosis (arrow) of the anal fin of a Chinook salmon (*O. tshawytscha*) infected with *F. columnare*. Note also the yellowish discoloration of the lesion due to the presence of the yellow-pigmented bacterium, (d) severe necrosis of the gill lamellae (arrow), also accompanied by yellowish discoloration, of a Chinook salmon from which *F. columnare* was recovered, (e) multifocal ulceration (arrow) and hemorrhage on the dorsum of a rainbow trout infected with a previously uncharacterized *Flavobacterium* sp (note faint yellowish discoloration to the right of the arrow), (f) erosion and necrosis (arrow) of the adipose fin, which is accompanied by a yellowish discoloration, of a Chinook salmon infected with a previously uncharacterized flavobacterial species.

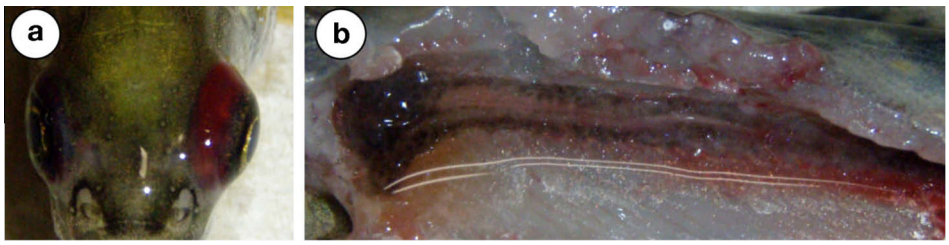

Fig. 3 Gross pathology in fish that were experimentally challenged with previously undescribed *Flavobacterium* spp. isolates that were originally recovered from Great Lakes fishes. (a) Severe unilateral exophthalmia with diffuse periocular hemorrhage in a *Flavobacterium* sp. S21-infected brook trout fingerling, (b) pale, mottled, hemorrhagic, and edematous kidney of a *Flavobacterium* sp. S21-infected brook trout fingerling.

Chryseobacterium spp. are inherently resistant to a wide spectrum of antibiotics, including tetracyclines, erythromycin, linezolid, polymyxins, aminoglycosides, chloramphenicol, and many β-lactams, while also being intermediately sensitive to vancomycin and clindamycin and vary in their sensitivity to trimethoprim-sulfamethoxazole [165,214,227,228]. Michel et al. [229] found that among the 65 isolates they obtained from aquatic habitats, 89% were resistant to polymyxin-B, 97% were resistant to ampicillin, 62% were resistant to erythromycin, and 54% were resistant to oxytetracycline, while 21.5% and 41.5% were resistant and moderately resistant to florfenicol. Additionally, 69% of the isolates were sensitive to trimethoprim-sulfamethoxazole.

Emergence of flavobacteria in the Laurentian Great Lakes

Recently, multiple wild fish kills in the Great Lakes Basin have been attributed to infectious agents, such as viral hemorrhagic septicemia virus VHSv; [230], *Renibacterium salmoninarum* [231], and *F. columnare* (Faisal and Loch unpublished). Indeed, as a group, flavobacteria have historically accounted for more fish mortality in the hatcheries of the State of Michigan than all other pathogens combined [232,233], though it was unclear which *Flavobacterium* spp. were involved. In this context, the Michigan Department of Natural Resources (MDNR) and the Aquatic Animal Health Laboratory at Michigan State University (MSU-AAHL) undertook extensive field and laboratory studies to identify pathogens threatening conservation efforts in wild fish stocks, as well as those reared within State Fish Hatcheries.

These studies revealed that a diverse assemblage of *Flavobacterium* and *Chryseobacterium* spp. were associated with diseased, as well as apparently healthy wild, feral, and farmed Great Lakes fishes [25,26,234–236]. Among 254 fish-associated flavobacterial isolates that were recovered from 21 fish species during 2003–2010, 211 were identified as *Flavobacterium* spp. and 43 as *Chryseobacterium* spp. according to partial 16S rRNA gene sequencing and phylogenetic analysis [235]. A dendrogram depicting this diversity is displayed in Fig. 1. Although *F. psychrophilum* and *F. columnare* were associated with multiple fish mortality events and severe gross pathology (Fig. 2a–d), many previously uncharacterized flavobacteria were recovered from systemically infected fish showing overt signs of disease (Fig. 2e and f). Indeed, the majority of the isolates were either most similar to recently described fish-associated *Flavobacterium* and *Chryseobacterium* spp. whose presence had never before been reported in North America (e.g., *F. oncorhynchi*, *F. araucananum*, *C. viscerum*, *C. piscicola*, and *C. chaponense*), or they were phylogenetically distinct from all of the described species and were suspected as comprising novel flavobacterial

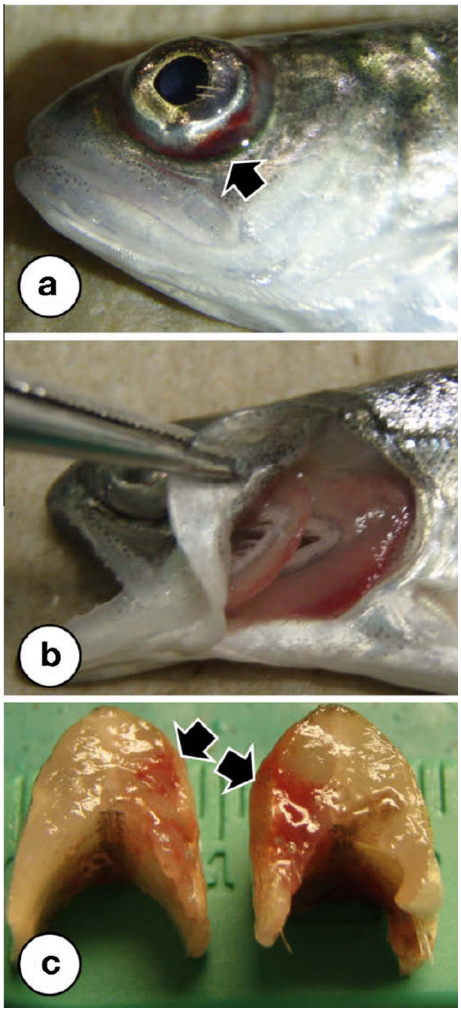

Fig. 4 Gross lesions observed in fish experimentally challenged with *Chryseobacterium* spp. isolates. (a) Unilateral exophthalmia and periocular hemorrhage (arrow) in a Chinook salmon fingerling infected with *Chryseobacterium* sp. T31, (b) pale hemorrhagic gills of a Chinook salmon challenged with *Chryseobacterium* sp. T28, (c) extensive multifocal hemorrhage (arrows) within the musculature of a *Chryseobacterium* sp. T72 infected Chinook salmon.

taxa [235]. Interestingly, 144 of the 254 flavobacterial isolates that were characterized in that study were recovered from fish species that were either accidentally or intentionally introduced into the Great Lakes.

Further study of a subset (n = 65) of these Great Lakes fish-associated *Flavobacterium* spp. using 16S rRNA gene sequencing and phylogenetic analyses based upon neighbor-joining and Bayesian methodologies revealed that 13 of these isolates were highly similar to the newly described fish-associated *F. plurextorum*, *F. spartansii*, and *F. tructae* [236]. However, the remaining isolates did not conclusively match any described *Flavobacterium* spp. and were thus suspected as representing novel flavobacterial species. Further polyphasic characterization, which included morphological, physiological, biochemical, and additional phylogenetic analyses, was undertaken on 6 isolates that represented a range of genetic distinctness and association with disease signs in hatchery raised or free-ranging fish [236]. Those findings showed that at least five of the six isolates were most likely novel *Flavobacterium* species that had never before been reported from fish. The same study confirmed that some of these flavobacteria were capable of generating gross pathology in experimentally challenged fish (Fig. 3), where flavobacteria were re-isolated from their brains, spleens, livers, and kidneys [236]. Similarly, median lethal dose (LD_{50}) experiments with two isolates of *F. spartansii* ($T16^T$ and S12) indicated that this bacterium is a facultative pathogen of its host of origin, the Great Lakes Chinook salmon, which is capable of inducing mortality and histopathological changes that include severe proliferative branchitis, lymphocytic and histiocytic myositis, multifocal hepatic necrosis, lymphocytic hepatitis, renal tubular degeneration and necrosis, and multifocal edema within the granular cell layer of the cerebellar cortex [237].

A similar study was conducted with the Great Lakes fish-associated *Chryseobacterium* spp. [238], which showed that multiple isolates were highly similar to isolates previously recovered from Europe, Africa, and Asia, but were never before been reported in North America. Polyphasic characterization, which also included fatty acid profiling, highlighted the diversity of Great Lakes' fish-associated *Chryseobacterium* spp. and also suggested that at least two taxa represented potentially novel *Chryseobacterium* spp. Experimental challenges with representative *Chryseobacterium* spp. isolates showed they were capable of inducing varying degrees of gross pathology (Fig. 4) in multiple Great Lakes salmonids, some of which were severe and resulted in host death. LD_{50} experiments for one isolate (e.g., *Chryseobacterium* sp. T28) demonstrated that the LD_{50} exceeded 4.5×10^8 cfu, indicating that it is a facultative salmonid-pathogen [238]. An investigation into the histopathological changes that accompanied T28 infection revealed epithelial hyperplasia of the secondary lamellae and interlamellar space that resulted in secondary lamellar fusion and monocytic infiltrate and mucus cell hyperplasia within primary lamellae, which are consistent with a proliferative branchitis, along with monocytic myositis, hemorrhage within the muscle, liver, adipose tissue, and ovaries, and hepatic and renal degenerative changes [238]. Median lethal dose experiments with *C. aahli* $T68^T$ demonstrated it was mildly pathogenic to yearling brook trout in terms of induced mortality; however, histopathological changes in infected fish were at times severe, and included a proliferative branchitis, monocytic epicarditis, pancreatitis, hepatitis, and peritonitis, multifocal hemorrhage within the muscle and adipose tissue, splenic congestion and hemosiderosis, and necrosis of the interstitial tissue within the posterior kidney [237].

Conclusions and future research

Research on flavobacteria is essential due to their role as important etiological agents of disease and their importance in microbial ecology. However, researchers working with these organisms are faced with significant challenges. First, many flavobacteria, especially those that are pathogenic to fish, are fastidious and grow only on nutrient poor media [79] that, under many conditions, must be supplemented with a variety of antibiotics to prevent overgrowth by other less fastidious bacteria. The ratio and brand of ingredients incorporated into these media, as well as osmotic conditions, can affect the ability to cultivate some flavobacteria [239–241]. Even when an ideal culture medium for a particular situation is used, the slow generation time of some species and variety in preferred incubation conditions (e.g., temperature, aerobic vs. microaerophillic atmosphere) can impede successful culture of all organisms.

A second challenge, which was alluded to above, is the rapid pace at which members of the family Flavobacteriaceae are discovered and described. Indeed, one must constantly search the literature to include species that must be incorporated into any identification scheme and to stay abreast with numerous changes in nomenclature [210,242]. A third challenge, especially when working with fish-associated flavobacteria, is their ubiquity in aquatic habitats and on/in the skin, gills, mucus, and intestines of fish [79]. While some of the flavobacteria that are present on apparently healthy fish have also been implicated as facultative fish pathogens (i.e., *F. hydatis*, *F. succinicans*, *C. scophthalmum*; [8,9,219]), the role that many flavobacteria play in fish health is not well understood. It may not be clear whether external flavobacteria are transient inhabitants of fish or whether they are normal constituents of their bacterial flora and which of the newly described species are truly pathogens. Indeed, some of the bacterial flora that are present in the skin, gills, and intestine of fish inhibited known fish pathogens [243,244], while some *Chryseobacterium* strains present on the skin of salamanders (*Hemidactylium scutatum*) exhibit antifungal activity [245] and thus may play a mutualistic role with their host.

Fourth, there is a lack of specific diagnostic reagents available to detect and identify many fish-associated flavobacteria outside of those that are commonly associated with fish disease. For example, the majority of recent reports of novel or less common fish-associated flavobacteria have based their identification on extensive polyphasic characterization as outlined by Bernardet et al. [69]. These methods work well for a definitive identification and allow for a consistent comparison among the described flavobacteria, but they necessitate culture of the organism and the technical capabilities to perform an array of biochemical and molecular assays, some of which are specialized (e.g., DNA-DNA hybridization, fatty acid methyl ester analyses).

Finally, another impediment to flavobacterial research, especially fish-pathogenic flavobacteria, is the difficulty

associated with experimental challenge models to study their pathogenicity. Despite the fact that *F. psychrophilum* causes economically depressing disease outbreaks in fish all over the world and has been studied extensively, a truly reliable experimental model to assess its pathogenicity still does not exist. For example, experimental infections conducted by numerous authors utilizing immersion challenges have yielded highly variable results and highlight that bath infections are difficult to control, standardize, and reproduce [97,246–251]. However, Madsen and Dalsgaard [247] studied the reproducibility of *F. psychrophilum* challenge methods and showed that, for the most part, intraperitoneal injection of the bacterium was reproducible, though some parameters (i.e., isolate used, number of fish within the tank, origin of fish, weight of fish) could introduce variability. Thus, recent research aimed at elucidating the virulence mechanisms and pathogenesis of *F. psychrophilum*, as well as ways to prevent and treat the diseases it causes, now use intraperitoneal [252–254], subcutaneous [255], or intramuscular injection [256–258], even though it is recognized that these methods bypass important aspects of the innate immune system.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Acknowledgments

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