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Flavobacterium oncorhynchi sp. nov., a new species isolated from rainbow trout (*Oncorhynchus mykiss*)☆

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ABSTRACT

Eighteen isolates of a Gram-negative, catalase and oxidase-positive, rod-shaped bacterium, recovered from diseased rainbow trout (*Oncorhynchus mykiss*), were characterized, using a polyphasic taxonomic approach. Studies based on comparative 16S rRNA gene sequence analysis showed that that the eighteen new isolates shared 99.2–100% sequence similarities. Phylogenetic analysis revealed that isolates from trout belonged to the genus *Flavobacterium*, showing the highest sequence similarities to *F. chungangense* (98.6%), *F. frigidimaris* (98.1%), *F. hercynium* (97.9%) and *F. aquidurense* (97.8%). DNA–DNA reassociation values between the trout isolates (exemplified by strain 631-08^T) and five type strains of the most closely related *Flavobacterium* species exhibited less than 27% similarity. The G + C content of the genomic DNA was 33.0 mol%. The major respiratory quinone was observed to be menaquinone 6 (MK-6) and iso-$C_{15:0}$, $C_{15:0}$ and $C_{16:1}$ $\omega 7c$ the predominant fatty acids. The polar lipid profile of strain 631-08^T consisted of phosphatidylethanolamine, unknown aminolipids AL1 and AL3, lipids L1, L2, L3 and L4 and phospholipid PL1. The novel isolates were differentiated from related *Flavobacterium* species by physiological and biochemical tests. On the basis of the evidence from this polyphasic study, it is proposed that the isolates from rainbow trout be classified as a new species of the genus *Flavobacterium*, *Flavobacterium oncorhynchi* sp. nov. The type strain is 631-08^T (=CECT 7678^T = CCUG 59446^T).

Introduction

The genus *Flavobacterium* accommodates Gram-negative, non-spore-forming, aerobic, oxidase-positive, non-fermenting, predominantly gliding, yellow-pigmented bacteria that have menaquinone-6 as the primary respiratory quinone [1,4]. The genus has undergone a considerable expansion in the last decade; currently the genus *Flavobacterium* comprises 75 species with validly published names at the time of writing [11]. Due to their physiological diversity, they have been isolated from a range of ecological niches, including freshwater and saltwater ecosystems [4]. A number of *Flavobacterium* spp. are pathogenic or are regarded as opportunistic pathogens, causing disease in a wide variety of organisms, including plants, fish and humans [4,16,19]. One of the major concerns regarding some members of this genus is their ability to cause disease in aquaculture settings. Thus, several species, including *Flavobacterium psychrophylum*, *Flavobacterium johnsoniae*, *Flavobacterium branchiophilum* and *Flavobacterium columnare* have been associated with clinical diseases in fishes and represent major threats to commercial aquaculture worldwide [4,8,12,13]. Other species of *Flavobacterium* such as *Flavobacterium aquatile*, *Flavobacterium hydatis* and *Flavobacterium succinicans* also are isolated occasionally from diseased fish [1,4,9].

In the present study, identification of isolates, recovered during the period of 2008–2009, from diseased juvenile rainbow trout (*Oncorhynchus mykiss*) presenting clinical symptoms indicative of *F. psychrophylum* infection was accomplished using phenotypic and genotypic techniques.

Materials and methods

Isolation, morphological, physiological and biochemical characteristics

Diseased trout were sent (alive) to the Centro Visavet at the Veterinary School in Madrid, Spain, for routine bacteriological analysis.

☆ The GenBank accession number for the 16S rRNA gene sequence of strains 631-08^T, 646B-08 and 666-09 are FN669776, FR870076 and FR870077, respectively.

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Samples from internal organs (spleen, liver, and kidney) and gills were spread plated on Anacker and Ordal agar [4] and incubated at 22 °C for 7 days. Bacterial colonies displaying a yellow color, characteristic for flavobacteria, were selected for further analysis. Eighteen Gram-negative rod-shaped organisms were recovered from the liver (628-1-08, 631-08^T, 646-08, 650-08, 662-09, 666-09) and gills (425B-08, 426B-08, 433B-08, 437B-08, 441B-08, 646B-08, 688B-08, 695B-08, 947B-08, 950B-08, 22B-09, 47B-2-09). The yellow pigmented colonies of the isolates on Anacker and Ordal agar [4] and their Gram-staining characteristic were consistent with the presumptive diagnosis of infection by *F. psychrophylum*. However, attempts to confirm the identification of the bacteria from the diseased trout as *F. psychrophilum*, using a species-specific PCR assay [31], were unsuccessful; none of the 18 isolates gave the expected amplicon of 1089 bp, characteristic of this bacterial fish pathogen. For further studies, isolates were cultured on tryptone glucose extract agar (Difco) and incubated at 22 °C for 72 h under aerobic conditions. Stock cultures of the isolates were stored at −40 °C.

The standards for the description of new taxa in the family *Flavobacteriaceae* were followed for phenotypic characterization [2]. A number of key characteristics were performed, using standard procedures [5,6,27], i.e., Gram-staining, production of catalase and oxidase, and hydrolysis of agar, casein, L-tyrosine, aesculin, DNA, urea, gelatin and starch. Growth in brain heart infusion broth was assessed at 15, 25, 30, 37 and 42 °C, with 3.0, 4.5 and 6.5% added NaCl, and under anaerobic (with 4–10% CO_2) and microaerobic (with 5–15% O_2 and 5–12% CO_2) conditions, using GasPak Plus and CampyPak Plus sytems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco), trypticase-soy (bioMérieux) and marine (Difco) agars. The presence of gliding motility and the production of flexirubin-type pigments and extracellular glycans were investigated as described previously [2]. The strains were further biochemically characterized, using the API 20NE systems (bioMérieux) according to the manufacturer's instructions, except that the incubation temperature for API 20NE was 22 °C. The type strains of species *Flavobacterium chungangense* CCUG 58910^T, *Flavobacterium frigidimaris* CCUG 59364^T, *Flavobacterium hercynium* CCUG 59448^T, *Flavobacterium aquidurense* CCUG 59847^T and *Flavobacterium resistents* CCUG 59848^T were used as reference strains for the investigation of the phenotypic properties of trout isolates under the same laboratory conditions.

Isolate 631-08^T has been deposited in the Spanish Type Culture collection (CECT) and in the Culture Collection of the University of Gothenburg (CCUG) Sweden, under the accession numbers CECT 7678^T and CCUG 59446^T, respectively. Isolates 666-09 and 646-08 were also deposited in the CECT and CCUG under the accession numbers CECT 7848 and CCUG 59447 and CECT 7792 and CCUG 59310, respectively

Cell fatty acid compositions, respiratory quinone and polar lipids analyses

Strains 631-08^T (CCUG 59446^T), 646-08 (CCUG 59310) and 666-09 (CCUG 59447) and the type strains of species analysed for comparison (Table 1) were cultivated aerobically, on Columbia II agar base (BBL 4397596) with 5% horse blood, at 30 °C, and harvested after 30 h ± 2 h. Cell fatty acid-fatty acid methyl ester (CFA-FAME) analyses were performed, using a protocol similar to that of the MIDI Sherlock MIS system [26]. Bacterial biomass was removed from the agar medium using a plastic inoculating loop, carefully scraped to avoid including medium in the sample; 50–100 mg of cells were transferred to glass tubes. The cells were saponified by mild alkaline methanolysis and released fatty acids were methylated followed by organic extraction CFAs were identified and quantified by gas-chromatography (Hewlett Packard HP 5890). Retention times of CFA peaks were converted to Equivalent Chain Length (ECL) values and the % area for each peaks was determined. The Agilent MIS FAME standard was used as reference for identification of peaks. The relative amount of each CFA in a strain was expressed as a percentage of the total fatty acids in the profile of a strain. Further details of the methodology can be found at http://www.ccug.se/pages/CFA_method_2008.pdf.

Determination of the respiratory quinone and polar lipids of the type strain (631-08^T) was carried out by the identification service of the DSMZ and Dr. B.J. Tindall (DSMZ, Braunschweig, Germany).

16S rRNA gene sequencing, DNA–DNA hybridization and DNA G + C content

The phylogenetic affinity of the isolates was established by sequencing of their 16S rRNA gene, as described previously [29]. A continuous segment (approximately 1395 bases) of the 16S rRNA gene of three isolates (433B-08 and 441B-08 and 631-08^T) and 1000 nucleotides from the other fifteen isolates were determined from PCR-amplified products, derived from universal primers pA (5′-AGAGTTTGATCCTGGCTCAG; positions 8-27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA; positions 1541–1522. Sequence searches of GenBank were performed, using the program FASTA [23]. Sequences of the most closely related species and other representative species within the genus *Flavobacterium* were retrieved from GenBank and aligned with the newly determined sequences, using the program, SeqTools [23]. Phylogenetic trees were constructed according to three different algorithms: Neighbour-Joining [25], using the programs SeqTools and TREEVIEW [22,24], Maximum-Likelihood, using the PHYML software [14]; and Maximum-Parsimony, using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 [18]. Genetic distances for the Neighbour-Joining and the Maximum-Likelihood algorithms were calculated by the Kimura two-parameter [17] and close-neighbour-interchange (search level = 2, random additions = 100) was applied in the Maximum-Parsimony analysis. The stabilities of the groupings were estimated by bootstrap analysis (1000 replications).

DNA–DNA hybridization experiments were carried out between three isolates (631-08^T, 646B-09, 666-09) and between strain 631-08^T and their nearest phylogenetic neighbours, *F. chungangense* CCUG 58910^T, *F. frigidimaris* CCUG 59364^T, *F. hercynium* CCUG 59448^T, *F. aquidurense* CCUG 59847^T and *F. resistens* CCUG 59848^T. DNA was extracted and purified by the method of Marmur [20] and genomic DNA–DNA reassociation analysis was carried out, using hybridization protocols described by Urdiain et al. [28]. Two independent determinations were carried out for each experiment. The results reported are mean values.The G + C content of the DNA of a representative isolate (strain 631-08^T) was determined from the mid-point value (T_m) of the thermal denaturation profile [21] obtained with a Perkin-Elmer UV–Vis Lambda 20 spectrophotometer at 260 nm.

PFGE typing

All 18 trout isolates were characterized by pulsed-field gel electrophoresis (PFGE) profiling of their genomic DNA, after digestion with the restriction enzyme XhoI, according to the specifications of Chen et al. [7], to recognize potential prevalent clones. Similarities between restriction endonuclease digestion profiles were based on visual comparison of the band patterns of isolates run in the same gel by the use of the BioNumerics software (version 3.0; Applied Maths, Kortrijk, Belgium) for comparisons. Strains were considered different when they differed in at least one band [10].