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Chryseobacterium tructae sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Three pale-orange bacteria (strains 1083-08, 1084-08^T and 1095B-08) were isolated from diseased rainbow trout. The isolates were Gram-staining-negative, catalase- and oxidase-positive, rod-shaped cells. Analyses of their 16S rRNA gene sequences confirmed their adscription to the genus *Chryseobacterium*. The three isolates shared 100% 16S rRNA gene sequence similarity and 98.5% similarity with *Chryseobacterium indologenes* CCUG 14556^T, being the closest phylogenetically related species. Genomic DNA–DNA hybridization similarity values between the three isolates were 94–100% and 2–39% between strain 1084-08^T and the type strains of other related *Chryseobacterium* species, confirming that the isolates represent a novel species within the genus *Chryseobacterium*. The DNA G+C content of the species was 33.6–36.1 mol%. The predominant respiratory quinone of strain 1084-08^T was MK-6 and the major fatty acids were iso-$C_{15:0}$, iso-$C_{17:1}$ $\omega 9c$, iso-$C_{17:0}$ 3-OH and $C_{16:1}$ $\omega 6c$. The isolates were distinguished from related *Chryseobacterium* species by a number of phenotypic properties. Based on the phenotypic, genotypic and phylogenetic findings, it is proposed that the new isolates from rainbow trout be classified as a new species of the genus *Chryseobacterium*, with the name of *Chryseobacterium tructae* sp. nov. The type strain is 1084-08^T (=CECT 7798^T = CCUG 60111^T).

Introduction

The genus *Chryseobacterium* is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, and represents one of the genera with a rapidly increasing number of species. At the time of writing, the genus *Chryseobacterium* comprised 59 described and validly published species [11]. Members of this genus have been isolated from a variety of environmental sources, including soil, water, sludge, plants, food products such as fish, meat, poultry, milk and lactic acid beverages, and human clinical specimens [5,6]. The significance of *Chryseobacterium* in veterinary medicine is primarily limited to some species, such as *C. balustinum*, *C. scophtalmum*, *C. arothri* (*currently C. hominis*) and *C. joostei*, that have been isolated from diseased fish [4,8,13,14,21,22]. Furthermore, three new species associated with fish disease have been described recently. *C. piscicola* was isolated from external lesions of diseased farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) [14], *C. chaponense* from external lesions, gills and fins of diseased Atlantic salmon [16] and *C. oncorhynchi* from gills and kidney of diseased rainbow trout [34].

In this article, we report the phenotypic, genotypic and phylogenetic characterization of three novel *Chryseobacterium*-like strains isolated from trout. Based on the presented findings, a new species of the genus *Chryseobacterium*, *Chryseobacterium tructae* sp. nov., is proposed.

Materials and methods

Isolation of bacteria

During the routine microbiological diagnosis from different clinical specimens of rainbown trout (*O. mykiss*) submitted to the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain), three novel Gram-negative, rod-shaped bacteria were recovered from liver, strains 1083-08 (CCUG 60110) and 1084-08^T (CCUG 60111^T), and gills (strain 1095B-08)

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of three different trout with a presumptive diagnosis of septicemia. The isolates were recovered on the same date, from two different ponds (1083-08 and 1084-08 from one pond and 1095B-08 from another pond) in the same fish farm. The strains were isolated on tryptone glucose extract agar (TGE; Difco) after incubation at 25 °C for 72 h under aerobic conditions.

16S rRNA gene sequence determinations and analyses

In order to establish the phylogenetic allocation of the bacteria, the 16S rRNA gene sequences of the three isolates were determined, as described previously [34], and subjected to a comparative analysis. A large continuous sequence (approximately 1400 bases) of the 16S rRNA gene of the three isolates was obtained in both directions, using universal amplification primers: pA (5′-AGAGTTTGATCCTGGCTCAG, positions 8–27, *Escherichia coli* numbering); and pH* (5′-AAGGAGGTGATCCAGCCGCA, positions 1541–1522, *E. coli* numbering). The identifications of the phylogenetic neighbors and calculations of pair-wise 16S rRNA gene sequence similarities were achieved, using the EzTaxon server (http://www.eztaxon.org/) [9]. The 16S rRNA gene sequences of the type strains of all species with valid names of the genus *Chryseobacterium* were retrieved from GenBank and aligned with the newly determined sequences using the program SeqTools [24]. Phylogenetic trees were constructed according to three different algorithms: neighbor-joining [26], using the programs SeqTools and TREEVIEW [23,24]; maximum-likelihood, using the PHYML software [12]; and maximum-parsimony, using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 [18]. Genetic distances for the neighbor-joining and the maximum-likelihood algorithms were calculated by the Kimura two-parameter [17] and close-neighbor-interchange (search level = 2, random additions = 100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications).

Genomic DNA–DNA similarity determinations

Genomic DNA–DNA hybridizations were carried out between the three isolates (strains 1084-08^T, 1083-08 and 1095B-08) and between strain 1084-08^T and the type strains of all species with 16S rRNA gene sequence similarities greater than 97.0%. DNA was extracted and purified by the method of Marmur [19]. Hybridization studies were carried out, using the membrane method of Johnson [15], described in detail by Arahal et al. [1]. The hybridization experiments were carried out under optimal conditions, at a temperature of 44 °C, which is within the limits of validity for the membrane method [10]. The percentages of hybridization similarities were calculated as described by Johnson [15]. Three independent determinations were carried out for each experiment and the results reported as mean values.

DNA G+C content determination

The G+C contents of the genomic DNA of a representative isolate (strain 1084-08^T) and the other two isolates were determined from the mid-point value (T_m) of the thermal denaturation profile [20], obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm.

Fatty acid composition and respiratory quinone determinations

Respiratory quinones of strain 1084-08^T were extracted from 100 mg of freeze-dried cell material, using the two stage method described by Tindall [29,30], and further separated by thin layer chromatography on silica gel and analyzed, using HPLC, by the identification service of the DSMZ (Braunschweig, Germany).

The cell fatty acid–fatty acid methyl ester (CFA–FAME) analyses of strain 1084-08^T was done with cultures on Columbia II agar base (BBL 4397596) with 5% horse blood, at 30 °C for 30–48 h, under aerobic conditions. The CFA–FAME profile was determined, using gas chromatography (Hewlett Packard HP 5890) and a standardized protocol similar to that of the MIDI Sherlock MIS system [27], described previously [34]. CFAs were identified and the relative amounts were expressed as percentages of the total fatty acids of the strain.

Physiological and biochemical characterization

The minimal standards for the description of new taxa in the family *Flavobacteriaceae* [3] were followed for the phenotypic characterization of the isolates. Gram-staining was performed as described by Smibert and Krieg [28]. Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on filter paper and catalase activity was determined, using 3% H_2O_2 solution [28]. Hydrolysis of Tween 80 (1%, v/v), L-tyrosine (0.5%, w/v), lecithin (5%, w/v) [28], esculin (0.01% esculin and 0.05% ferric citrate, w/v), gelatin (4%, w/v), starch (0.2%, w/v), and casein [50% skimmed milk (Difco), v/v] were tested using nutrient agar [28,31]. DNase test agar (Difco) was used for the DNase assay. Hydrolysis of urea (1%, w/v) was tested as described by Bowman et al. [7]. 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 micro-aerobic (with 5–15% O_2 and 5–12% CO_2) conditions, using GasPak Plus and CampyPak Plus systems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco) and trypticase-soy (bioMérieux) agar plates. The presence of gliding motility, using the hanging drop technique, the production of flexirubin-type pigments and extracellular glycans were assessed, using the KOH and Congo red tests, respectively [3]. The strains were further biochemically characterized using the API 20NE and API Zym systems (bioMérieux) according to the manufacturer's instructions, except that incubation temperature was 25 °C. The type strains of species *C. oncorhynchi* 701-08^T, *C. indologenes* CCUG 14556^T, *C. ureilyticum* CCUG 52546^T, *C. gleum* CCUG 14555^T, *C. arthrosphaerae* CCUG 57618^T, *C. jejuense* CCUG 61058^T, *C. hominis* CCUG 52711^T, *C. shigense* CCUG 61059^T, *C. aquifrigidense* CCUG 61061^T and *C. joostei* CCUG 46665^T were included as references for the investigation of the phenotypic properties of the trout isolates under the same laboratory conditions.

PFGE typing

The 3 isolates from diseased trout were characterized by pulsed-field gel electrophoresis (PFGE) profiling of their genomic DNAs, according to the specifications of Zamora et al. [34], with the exception that the restriction enzymes were *Xba*I *and Bcu*I (MBI Fermentans), and the pulse times ranged from 0.1 to 15 s for a period of 30 h. Similarities between restriction endonuclease digestion profiles were based on visual comparisons of the band patterns of isolates run in the same gel. Strains differing in at least one band were considered different.

Results and discussion

Comparative 16S rRNA gene sequence analysis revealed 100% similarity between the sequences of the three isolates from diseased trout, suggesting an identical or close genealogical identity. Sequence searches showed that the isolates were most closely related to members of the genus *Chryseobacterium*. The 16S rRNA