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# *Flavobacterium ceti* sp. nov., isolated from beaked whales (*Ziphius cavirostris*)

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Three isolates of a Gram-negative, catalase- and oxidase-positive, rod-shaped bacterium, isolated from the lung and liver of two beaked whales, were characterized by phenotypic and molecular genetic methods. Based on cellular morphology and biochemical criteria, the isolates were tentatively assigned to the family *Flavobacteriaceae*, although they did not appear to correspond to any recognized species. Comparative 16S rRNA gene sequencing showed that the three new isolates shared 100 % sequence similarity. The unknown bacterium was phylogenetically closely related to, but distinct from the type strains of *Flavobacterium johnsoniae* (93.7 % sequence similarity), *Flavobacterium frigidimaris* (93.4 %), *Flavobacterium aquidurense* (93.4 %), *Flavobacterium hibernum* (93.4 %) and *Flavobacterium degerlachei* (93.4 %). The novel isolates were readily distinguished from these and other related *Flavobacterium* species by physiological and biochemical tests. On the basis of phenotypic and phylogenetic evidence, it is proposed that the unknown isolates from whales are classified as a novel species of the genus *Flavobacterium*, *Flavobacterium ceti* sp. nov. The type strain is 454-2$^T$ (=CECT 7184$^T$ =CCUG 52969$^T$).

The genus *Flavobacterium* (Bergey *et al.*, 1923; Bernardet *et al.*, 1996; Bernardet & Bowman, 2006) is the type genus of the family *Flavobacteriaceae*, which currently comprises more than 25 other genera (Bernardet *et al.*, 1996; Bernardet & Nakagawa, 2006). Members of the genus *Flavobacterium* are widely distributed in nature, especially in cold marine environments (Bernardet & Bowman, 2006). Most species of *Flavobacterium* are harmless, but some are opportunistic or true pathogens. *Flavobacterium psychrophilum*, *Flavobacterium branchiophilum* and *Flavobacterium columnare* are fish-pathogenic species responsible for various diseases in salmonids and other fish species. *Flavobacterium hydatis* and *Flavobacterium succinicans* have also been isolated from diseased salmon, but the pathogenicity of these two species has not been clearly demonstrated (Bernardet & Bowman, 2006). Only one questionable case of human lung disease associated with *Flavobacterium* species has been reported (Bernardet & Bowman, 2006). In this study, we report the phenotypic and phylogenetic features of three bacterial strains isolated from two beaked whales.

The bacterial strains (designated 453-2, 453-5 and 454-2$^T$) were isolated from the liver (454-2$^T$ and 453-2) and lung (453-5) of two stranded beaked whales (*Ziphius cavirostris*) found along the coast of the Canary Islands (Spain). The two animals showed no clinical signs of disease and no apparent lesions were observed after post-mortem examination. Isolate 454-2$^T$ was recovered in pure culture, while the other two isolates were obtained in mixed culture with members of the genus *Vibrio*. Samples were collected and frozen at −40 °C until being processed in the laboratory. Strains were isolated on Columbia blood agar plates (bioMérieux) after incubation at 37 °C for 24 h under aerobic conditions.

The isolates were tested for a number of key characteristics by using standard procedures (Smibert & Krieg, 1994; Bowman *et al.*, 1996, Bowman, 2000), such as Gram staining, production of catalase and oxidase and hydrolysis of agar, alginate, casein, L-tyrosine, pectin, aesculin, CM-cellulose, DNA, urea, gelatin and starch. Growth in brain heart infusion broth was assessed at 4, 15, 22, 30, 37 and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 454-2$^T$ is AM292800.

  

42 °C, with 3, 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 systems (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 following the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). The strains were characterized biochemically using the API 20NE, API 20E and API ZYM systems (bioMérieux) according to the manufacturer's instructions, except that the incubation temperature for API 20NE was 37 °C. Using the miniaturized biochemical kits, the isolates displayed identical phenotypic profiles. A detailed description of the morphological, physiological and biochemical characteristics of the isolates is given in the species description and in Table 1.

To establish the phylogenetic affinities of the unknown isolates, their 16S rRNA gene sequences were determined as described previously (Vela *et al.*, 2005) and subjected to a comparative analysis. The almost-complete sequences (>1400 nucleotides) of the three strains were determined and pairwise analysis revealed that the 16S rRNA gene sequences of the strains were identical. Sequence searches of GenBank using the program FASTA (Pearson, 1994) showed that the isolates were most closely related to members of the family *Flavobacteriaceae*. The isolates exhibited the highest levels of 16S rRNA gene sequence similarity with the type strains of *Flavobacterium johnsoniae* (93.7 % sequence similarity), *Flavobacterium frigidimaris* (93.4 %), *Flavobacterium aquidurense* (93.4 %), *Flavobacterium hibernum* (93.4 %) and *Flavobacterium degerlachei* (93.4 %). These sequences and those of other known related strains were retrieved from GenBank and aligned with the newly determined sequences using the program DNATools (Rasmussen, 1995). Phylogenetic trees were constructed according to three different algorithms: neighbour-joining (Saitou & Nei, 1987) using the programs DNATools and TreeView (Page, 1996), maximum-likelihood using the PHYML software (Guindon & Gascuel, 2003) and maximum-parsimony using the software

**Table 1.** Characteristics that differentiate *Flavobacterium ceti* sp. nov. from related *Flavobacterium* species

Strains: 1, 454-2$^T$ (*F. ceti* sp. nov.); 2, *F. frigidimaris* KUC-1$^T$; 3, *F. aquidurense* (5 strains); 4, *F. saccharophilum* IAM 14309$^T$; 5, *F. hibernum* ACAM 376$^T$; 6, *F. gelidilacus* LMG 21477$^T$; 7, *F. hercynium* (5 strains); 8, *F. hydatis* IAM 12365$^T$; 9, *F. weaverense* AT1042$^T$; 10, *F. flevense* DSM 1076$^T$; 11, *F. degerlachei* (14 strains); 12, *F. daejeonense* GH1-10$^T$; 13, *F. johnsoniae* DSM 2064$^T$. Data from this study and from Bernardet *et al.* (1996), McCammon *et al.* (1998), Van Trappen *et al.* (2003, 2004), Nogi *et al.* (2005), Bernardet & Bowman (2006), Kim *et al.* (2006), Yi & Chun (2006) and Cousin *et al.* (2007). All taxa have MK-6 as major respiratory quinone. +, Positive reaction; −, negative ; (+), weakly positive ; V, variable; tr, traces (<1 % of total); ND, no data available.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Growth on: | | | | | | | | | | | | | |
| Nutrient agar | (+) | + | + | + | + | + | + | + | + | + | + | + | + |
| Trypticase soy agar | + | + | + | + | + | + | − | + | + | + | + | + | + |
| Growth at 37 °C | + | − | − | − | − | − | − | − | − | − | − | − | − |
| Tolerance of 3 % NaCl | + | + | − | − | − | + | − | − | + | ND | + | + | − |
| Gliding motility | − | + | − | + | + | − | + | + | − | + | − | − | + |
| Flexirubin-type pigments | + | + | − | + | + | − | − | + | − | − | − | − | + |
| Acid production from carbohydrates | − | + | ND | + | + | − | ND | + | + | + | − | + | + |
| Hydrolysis of: | | | | | | | | | | | | | |
| Gelatin | + | + | − | + | + | + | + | + | − | − | − | − | + |
| Starch | + | + | + | + | + | + | + | + | + | − | + | + | + |
| CM-cellulose | − | − | − | + | − | − | − | − | − | − | − | − | + |
| Agar | − | − | − | + | − | − | − | − | − | + | − | − | − |
| Alginate | − | ND | ND | + | − | − | ND | − | − | − | − | − | + |
| Aesculin | − | + | + | + | + | − | + | + | − | + | + | + | + |
| DNA | − | ND | − | − | + | − | − | + | − | − | − | − | + |
| β-Galactosidase activity | − | + | (+) | + | + | − | − | + | − | + | − | + | + |
| Nitrate reduction | − | − | V | + | + | − | + | + | − | − | − | + | + |
| Whole-cell fatty acids (%)* | | | | | | | | | | | | | |
| iso-$C_{15:0}$ | 45.9 | 26.7 | 15.1±1.1 | 27.3 | 18.6 | 2 | 23.0±0.1 | 31.6 | 3.2 | 14.5 | 5.8±2.2 | 23.0 | 24.9 |
| iso-$C_{16:0}$ | tr | tr | tr | 1.5 | 1.1 | 8 | tr | 1.0 | 8.6 | 1.4 | 3.9±0.9 | 1.1 | 3.4 |
| iso-$C_{16:0}$ 3-OH | tr | 1.3 | 2.3±0.1 | 2.1 | 2.9 | 10 | 1.1±0.1 | 1.1 | 14.8 | 3.8 | 9.7±1.8 | 2.1 | 4.4 |
| DNA G+C content (mol%) | 36.7 | 34 | 33.5 | 35.7 | 34 | 30 | 37.5 | 34 | 37 | 35 | 33.8–34.2 | 35 | 34 |

*Different growth conditions were used for analysis of the fatty acids of the strains listed.

package MEGA version 3.1 (Kumar *et al.*, 2004). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated by the Kimura two-parameter method (Kimura, 1980) and close-neighbour 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). The phylogenetic tree based on the neighbour-joining algorithm (Fig. 1) showed that the new isolates (as exemplified by strain 454-2$^T$) formed a separate branch that was further grouped with the recognized species of the genus *Flavobacterium*, which was supported by a bootstrap resampling value of 87 %. Strain 454-2$^T$ formed a peripheral branch in the *Flavobacterium–Myroides* cluster in the maximum-likelihood tree, while it was recovered with species of the genus *Myroides* in the maximum-parsimony tree. These phylogenetic relationships were not supported by bootstrap resampling (44 and 43 %, respectively). Moreover, the unknown isolates were excluded from the genus *Myroides* based on phenotypic considerations (Bernardet *et al.*, 1996; Vancanneyt *et al.*, 1996; Yoon *et al.*, 2006). For example, in contrast to members of the genus *Myroides*, the new isolates grew weakly under microaerobic conditions and failed to grow in broth containing 6.5 % NaCl and on MacConkey agar.

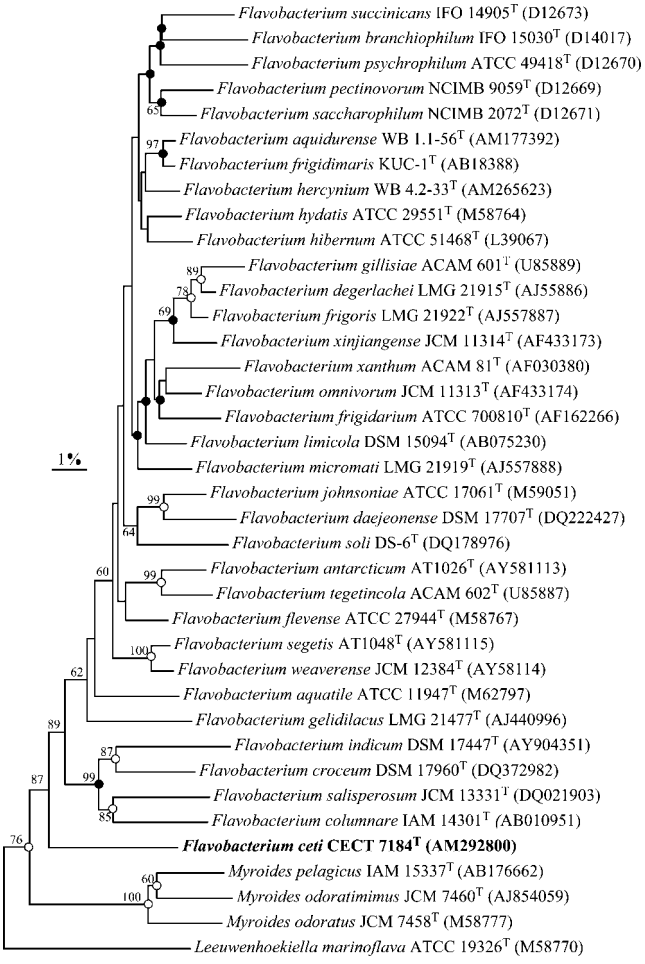


**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequence comparison obtained with the neighbour-joining algorithm showing the relationships of strain 454-2$^T$ to closely related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50 % are given at branching points. Solid circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood tree. Open circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood and parsimony trees. *Leeuwenhoekiella marinoflava* ATCC 19326$^T$ was used as an outgroup. Bar, 1 % sequence divergence.

Although the new isolates had a 16S rRNA gene sequence similarity lower than 94 % to members of the genus *Flavobacterium*, a value well below the cut-off recommended for delineation of genomic species (Wayne *et al.*, 1987), DNA–DNA hybridization experiments were carried out between the three isolates (strains 454-2$^T$, 453-2 and 453-5) and between strain 454-2$^T$ and its nearest phylogenetic neighbour, *F. johnsoniae* ATCC 17061$^T$. DNA was extracted and purified by the method of Marmur (1961). DNA–DNA hybridization studies were carried out by the membrane method of Johnson (1994), described in detail by Arahal *et al.* (2001). The hybridization experiments were carried out under optimal conditions, at a temperature of 49.5 °C, which is within the limits of validity for the filter method (De Ley & Tijtgat, 1970). The percentage of hybridization was calculated as described by Johnson (1994). Three independent determinations were carried out for each experiment and the results reported are mean values. The DNA–DNA hybridization study between the three new isolates showed 98–100 % DNA relatedness to each other, demonstrating that they are members of the same species (Wayne *et al.*, 1987). The DNA–DNA reassociation value between strain 454-2$^T$ and the type strain of *F. johnsoniae* (ATCC 17061$^T$) was 15 %, clearly confirming that the new isolates constitute a separate species (Wayne *et al.*, 1987).

The G+C content of the DNA was determined from the mid-point value ($T_m$) of the thermal denaturation profile (Marmur & Doty, 1962) obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The G+C content of the three isolates ranged from 36.4 to 37.2 mol%, values that were confirmed in three different assays. This DNA G+C range is consistent with that of members of the genus *Flavobacterium* (Bernardet & Bowman, 2006).

The determination of the respiratory quinone of one representative isolate (strain 454-2$^T$) was carried out by the identification service of the DSMZ (Braunschweig, Germany). The major quinone was MK-6, in accordance with all members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). Cellular fatty acid analysis of strain 454-2$^T$, grown on blood agar at 30 °C for 48 h under aerobic conditions, was performed as described by

Kämpfer & Kroppenstedt (1996). The major fatty acids of strain 454-2$^T$ were branched and hydroxy acids. The predominant fatty acids were iso-$C_{15:0}$ (45.9 %), iso-$C_{17:1}\omega9c$ (18.9 %), iso-$C_{17:0}$ 3-OH (11.6 %) and summed feature 4 ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH) (11.5 %). The isolate also contained moderate or small amounts of iso-$C_{15:0}$ 3-OH (3.7 %), $C_{15:0}$ (1.8 %) and an unknown fatty acid with an equivalent chain length of 13.566 (1.2 %). This fatty acid profile is in accordance with those of members of the genus *Flavobacterium* (Bernardet *et al.*, 1996, 2002; Bernardet & Bowman, 2006), although the proportions of some of the predominant fatty acids, such as iso-$C_{15:0}$, differ (Bernardet *et al.*, 1996) (Table 1).

Overall, the results of the present study show that the new isolates from whales constitute a distinct branch and do not display a close relationship with any described *Flavobacterium* species (Fig. 1). Moreover, the new isolates could be distinguished from their close phylogenetic relatives on the basis of phenotypic characteristics (Table 1). Therefore, on the basis of phylogenetic and phenotypic criteria, it is evident that the new isolates merit classification within a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium ceti* sp. nov. is proposed. Tests that are useful in differentiating *F. ceti* from related species sharing more than 93.0 % 16S rRNA gene sequence similarity are shown in Table 1.

### Description of *Flavobacterium ceti* sp. nov.

*Flavobacterium ceti* (ce′ti. L. n. *cetus* whale; L. gen. n. *ceti* of a whale).

Cells are Gram-negative rods, 0.3 μm wide and 2.0 μm long, non-spore-forming and non-gliding. Grows well under aerobic conditions, weakly under microaerobic conditions and poorly under anaerobic conditions. Grows at 22–37 °C, with optimal growth at about 35 °C, while no growth occurs at 42, 15 or 4 °C. Growth occurs on trypticase soy agar but not on MacConkey agar. Grows weakly on marine and nutrient agars after incubation at 37 °C for 48 h. Growth occurs in brain heart infusion broth containing 3 % NaCl but not with 4.5 or 6.5 % NaCl. Colonies are circular, orange-pigmented, smooth and entire on Columbia blood agar after 24 h of incubation at 37 °C. Colonies are non-haemolytic. Non-diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are not reduced. Gelatin, casein and starch are degraded, but CM-cellulose, alginate, pectin and agar are not. A brown pigment is produced on tyrosine agar, although degradation of tyrosine is not detected. Aesculin, arginine, DNA and urea are not hydrolysed. Acetoin, indole and $H_2S$ are not produced. Lysine and ornithine are not decarboxylated. Acid is not produced from D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, D-melibiose, amygdalin or L-arabinose. D-Glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate are not assimilated. Valine arylamidase, leucine arylamidase, alkaline phosphatase (weak reaction), esterase lipase C8 (weak reaction), acid phosphatase (weak reaction) and naphthol-AS-BI-phosphohydrolase (weak reaction) activities are present. Arginine dihydrolase, esterase C4, proline arylamidase, lipase C14, cystine arylamidase, trypsin, α-chymotrypsin, α-glycosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and ornithine decarboxylase activities are absent. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. Long-chain fatty acids are of the branched and hydroxy types, with iso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, iso-$C_{17:0}$ 3-OH and summed feature 4 ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH) predominating. The DNA G+C content is 36.4–37.2 mol%.

The type strain, 454-2$^T$ (=CECT 7184$^T$ =CCUG 52969$^T$), was isolated from the liver of a stranded beaked whale. The DNA G+C content of this strain is 36.7 mol%. Strains 453-2 (=CECT 7272) and 453-5 (=CECT 7271), also isolated from beaked whales, are also assigned to this species.

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