# *Leeuwenhoekiella blandensis* sp. nov., a genome-sequenced marine member of the family *Flavobacteriaceae*

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Bacteria in the family *Flavobacteriaceae* are increasingly recognized to play important roles in the degradation of organic matter during and following algal blooms. A novel heterotrophic, rod-shaped, aerobic, yellow-pigmented and gliding bacterium was isolated from a seawater sample collected in the Bay of Blanes in the north-western Mediterranean Sea. Analysis of its 16S rRNA gene sequence, retrieved from the whole-genome sequence, showed that the bacterium was closely related to members of the genus *Leeuwenhoekiella* within the family *Flavobacteriaceae*, phylum *Bacteroidetes*. Phenotypic, genotypic, chemotaxonomic and phylogenetic analyses supported the creation of a novel species to accommodate this bacterium, for which the name *Leeuwenhoekiella blandensis* sp. nov. is proposed. The type strain is MED 217$^T$ (=CECT 7118$^T$=CCUG 51940$^T$).

The phylum *Bacteroidetes* is one of the major components of marine bacterioplankton (Glöckner *et al.*, 1999; Kirchman, 2002), frequently accounting for approximately one-third of the bacteria in water of the world's oceans and seas. The *Flavobacteriaceae* is one of the main families in the *Bacteroidetes*, and members of the family effectively account for a large proportion of the members of the *Bacteroidetes* abundant in the marine environment (Abell & Bowman, 2005; Kirchman *et al.*, 2003).

Recent studies have suggested that bacteria belonging to the phylum *Bacteroidetes* play particularly important roles in the degradation of organic matter during and following algal blooms (Pinhassi *et al.*, 1999; Riemann *et al.*, 2000; Suzuki *et al.*, 2001). In seawater mesocosms in which the decay of phytoplankton was simulated by the addition of protein, Pinhassi *et al.* (1999) found a rapid growth response by a limited number of species in the phylum *Bacteroidetes*. Subsequent work measuring the uptake of radioactively labelled protein corroborated that bacteria of this phylum are well adapted to consuming and degrading protein (Cottrell & Kirchman, 2000).

In a recent experimental study of the identity of bacteria associated with different phytoplankton communities, Pinhassi *et al.* (2004) showed that flavobacteria are particularly responsive to phytoplankton blooms. In order to investigate whether the response of flavobacteria was a general phenomenon, their phylogenetic analysis included 16S rRNA gene sequences of representatives of the phylum *Bacteroidetes* from all published studies on the diversity of marine bacterioplankton associated with natural or experimental algal blooms (see Pinhassi *et al.*, 2004, and references therein). These studies encompassed blooms of diatoms, dinoflagellates as well as cyanobacteria. The analysis revealed that, even though the phylum *Bacteroidetes* is highly diverse, as many as 80 % of the *Bacteroidetes* sequences obtained (among a total of 63 sequences) belonged to one single family, the *Flavobacteriaceae*. Taken together, current data indicate that flavobacteria represent bacterial populations that are likely to mediate a substantial proportion of the carbon flow and nutrient turnover in the sea during and

Abbreviation: CM-cellulose, carboxymethylcellulose.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain MED 217$^T$ are DQ294290 and DQ294291 for two copies of the gene. The GenBank/EMBL/DDBJ accession number for the genome sequence is AANC00000000.

following algal blooms. An increased understanding of the diversity of the family *Flavobacteriaceae* is therefore important.

In the present study, we have characterized a novel flavobacterium, designated strain MED 217$^T$, isolated from a seawater sample. On the basis of a polyphasic approach (i.e. phylogenetic, phenotypic and genotypic analyses), we describe a novel member of the genus *Leeuwenhoekiella*.

Strain MED 217$^T$ was isolated from a surface seawater sample from the Bay of Blanes, in the north-western Mediterranean Sea (41° 40′ N 2° 48′ E) on the coast of Spain, collected on 23 May 2001. The sample was enriched with 0·6 μM $Na_2HPO_4$ (final concentration), and was incubated for 48 h at 16 °C in the dark. For strain isolation, 0·1 ml of a 100× dilution of sample water was spread onto ZoBell agar plates prepared from seawater from the Bay of Blanes (indicating an abundance of approximately $1 \times 10^3$ c.f.u. $ml^{-1}$). Strain MED 217$^T$ was one of the bacteria that exhibited an active growth response to phosphorus enrichment in this Mediterranean seawater, where typically bacterial growth is limited by the availability of phosphorus. After primary isolation and purification, strain MED 217$^T$ was cultivated at 23 °C on the same medium and stored at −80 °C in ZoBell's medium with 25 % (v/v) glycerol. For subsequent culturing of MED 217$^T$, marine broth/agar 2216 (Difco) was used, unless stated otherwise.

The presence of flexirubin pigments in strain MED 217$^T$ was determined by the method of Fautz & Reichenbach (1980). Determination of growth at different temperatures, requirement for NaCl for growth, production of acid from carbohydrates, nitrate reduction, production of hydrogen sulfide, indole and acetoin (Voges–Proskauer reaction), hydrolysis of casein, gelatin, starch, Tweens 20, 40 and 80, agar (1·5 %, w/v), DNA, urea and cellulose [carboxymethylcellulose (CM-cellulose) and filter paper] and oxidase, catalase, β-galactosidase and alkaline phosphatase activities were carried out according to standard procedures in bacteriology (Gerhardt, 1994). Utilization of carbon sources was determined as previously described (Nedashkovskaya *et al.*, 2003b). Hydrolysis of chitin (1 %, w/v) was determined by the appearance of clear zones around colonies on chitin agar. Gliding motility was evaluated as described by Bowman (2000). Susceptibility to antibiotics was examined by the disc-diffusion plate method, following the method of Nedashkovskaya *et al.* (2003a); in addition, discs containing chloramphenicol (30 μg), doxycycline (10 μg) and erythromycin (15 μg) were used. The API ZYM (bioMérieux) gallery was used for testing of enzyme activities of the strain according to the manufacturer's instructions. For the study of cell morphology, cells were grown at 21 °C in marine broth until early exponential phase (24 h incubation), when cells were fixed with glutaraldehyde and filtered onto polycarbonate filters of 0·2 μm pore size (Nuclepore). Samples were treated by sequential ethanol dehydration steps and critical-point drying with $CO_2$ and silver coating and viewed with a Hitachi S-3500N scanning electron microscope.

For analysis of the fatty acid composition, isolate MED 217$^T$, *Leeuwenhoekiella aequorea* LMG 22550$^T$ and *Leeuwenhoekiella marinoflava* LMG 1345$^T$ were grown on marine agar at 20 °C for 5 days until dense growth had developed. Cells were harvested and fatty acid composition was determined by quantitative GC and GC-MS procedures as described by Nichols *et al.* (1986, 1993).

Genomic DNA was extracted using the Marmur technique (Marmur & Doty, 1962). The DNA G+C content was determined by the thermal denaturation procedure (Sly *et al.*, 1986), using *Cellulophaga lytica* ATCC 23178$^T$ (DNA G+C content 33·0 mol%) and *Escherichia coli* K-12 ATCC 10798 (51·0 mol%) as reference strains. DNA–DNA hybridization was carried out by the spectrophotometric renaturation kinetics approach (Huß *et al.*, 1983), as later modified (Bowman *et al.*, 1998).

Whole-genome sequencing was carried out by the J. Craig Venter Institute through the Gordon and Betty Moore foundation initiative in Marine Microbiology (https://research.venterinstitute.org/moore/). The complete 16S rRNA gene sequences of MED 217$^T$ were 1515 and 1527 nt in length for two copies of the gene. A phylogenetic tree was constructed with reference sequences of members of the family *Flavobacteriaceae* as described by Bowman & McCuaig (2003), using Jukes–Cantor distances with the PHYLIP package (version 3.5) (Felsenstein, 1989) and the sequence of *Flexibacter flexilis* ATCC 23079$^T$ as the outgroup.

Strain MED 217$^T$ was a Gram-negative and chemoorganotrophic bacterium with respiratory-type metabolism and cells were motile by means of gliding. Cells appeared as single rods 0·4–0·7 μm in diameter and 1·5–4 μm in length; short chains were also observed. Cells of strain MED 217$^T$ divided by binary fission, although some showed polar constrictions resembling coccoid forms (Fig. 1), suggesting that cells may also form through budding fission, as previously reported for *Formosa agariphila* KMM 3901$^T$ (Nedashkovskaya *et al.*, 2006). The cell surface structures seen in Fig. 1 are most likely remnants of exopolysaccharides and/or precipitated salts. Other physiological and biochemical characteristics are given in the species description below and in Tables 1 and 2. Strain MED 217$^T$ is similar to recognized species in the genus *Leeuwenhoekiella* with respect to several phenotypic characteristics (Nedashkovskaya *et al.*, 2005). Nevertheless, specific features, including maximum temperature for growth, oxidation of D-galactose, D-glucose and DL-xylose, utilization of sucrose and mannitol and susceptibility to benzylpenicillin and tetracycline, distinguish MED 217$^T$ from its closest relatives (Table 1). The notably higher temperature for optimum growth of strain MED 217$^T$ may reflect that it was isolated from surface waters of the north-western Mediterranean Sea, which frequently reach high temperatures (25 °C or more during the summer), whereas *L.*

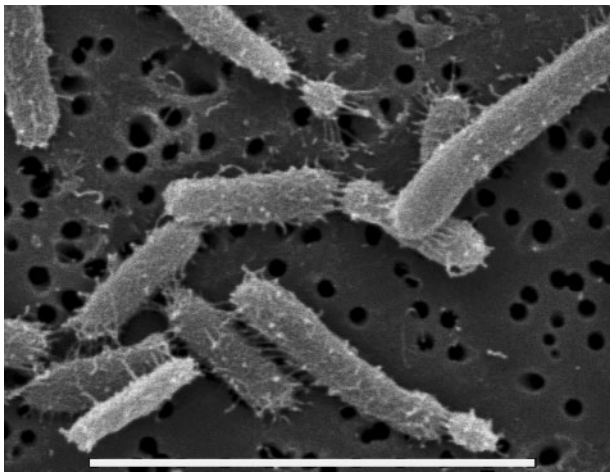

**Fig. 1.** Cells of strain MED 217$^T$ in the exponential growth phase (marine broth, 21 °C, 24 h). Scanning electron microscopy image of cells immobilized on polycarbonate filter of 0·2 μm pore size. Bar, 5 μm.

*aequorea* and *L. marinoflava* strains were recovered from the colder Antarctic and north-western Pacific Ocean marine environments, and from the North Sea, respectively (Colwell *et al.*, 1966; Tan *et al.*, 1999). In addition, the salinity optimum of MED 217$^T$ matches the range of salinities found at the site of its isolation in the Mediterranean Sea (36–39 ‰).

The whole-cell fatty acid composition of MED 217$^T$ was similar to those of *L. aequorea* and *L. marinoflava*, but with a lower proportion of the fatty acid 3-OH iso 17 : 0 (Table 2). The G+C content of the DNA of MED 217$^T$ was 42·5 mol%, as determined by the thermal denaturation method, a value higher than those of *L. aequorea* or *L. marinoflava* (Table 1). Whole-genome sequencing resulted in a G+C content of the DNA of 40 mol%. The former value was chosen for the species description, as comparative whole-genome sequence data on G+C content are still lacking for the majority of taxonomically described bacteria.

Genome sequencing showed that strain MED 217$^T$ has an annotated genome size of approximately 4·24 Mbp (3735 putative ORFs), which is relatively large compared with the genomes of other members of the *Flavobacteriaceae* that have been sequenced. These range in size from 2·74 Mbp (2557 putative ORFs) for *Polaribacter irgensii* to 3·87 Mbp (3498 putative ORFs) for the uncharacterized strain HTCC2170 (https://research.venterinstitute.org/moore/). The genome size of *L. marinoflava* was previously determined to be $2{\cdot}26 \times 10^9$ Da (Callies & Mannheim, 1980), which corresponds to approximately 4·20 Mbp. Thus, it seems that *Leeuwenhoekiella* species have a larger genome size than some other members of the *Flavobacteriaceae*.

Strain MED 217$^T$ has two copies of the 16S rRNA gene that share a sequence similarity of 98·3 % over the first 555 bp, after which the copies are identical (resulting in an overall sequence similarity of 99·5 %). Phylogenetic analysis of 16S rRNA gene sequence data showed that strain MED 217$^T$ clustered with bacteria belonging to the genus *Leeuwenhoekiella* (Fig. 2). Similarities of the two 16S rRNA gene sequences from strain MED 217$^T$ to the sequence of *L. aequorea* LMG 22550$^T$ were 96·1 and 96·7 % and similarities to the sequence of *L. marinoflava* LMG 1345$^T$ were 95·7 and 96·7 %. Sequence similarities to members of other genera of the *Flavobacteriaceae*, such as *Pibocella* and *Cellulophaga*, were consistently below 92 %. The level of DNA–DNA hybridization between strain MED 217$^T$ and *L. marinoflava* LMG 1345$^T$ averaged 21 % (±11 %, $n=5$).

Strain MED 217$^T$ showed distinct phenotypic and genotypic differences from the two recognized species of the genus

**Table 1.** Phenotypic properties of *Leeuwenhoekiella* species

Strains: 1, *L. blandensis* sp. nov. MED 217$^T$; 2, *L. aequorea* LMG 22550$^T$; 3, *L. marinoflava* LMG 1345$^T$. +, Positive; −, negative; V, variable. All strains gave positive results for the following: respiratory type of metabolism; motility by means of gliding; oxidase, catalase, β-galactosidase and alkaline phosphatase activities; growth with 0–15 % NaCl and at 37 °C; hydrolysis of casein, gelatin, starch and Tweens 20, 40 and 80; acid formation from glycerol; utilization of L-arabinose, D-glucose, D-lactose, D-mannose and sucrose; susceptibility to lincomycin, doxycycline, erythromycin and chloramphenicol; resistance to gentamicin, kanamycin, neomycin and polymyxin B. All strains were negative for the following: requirement for $Na^+$ ions for growth; nitrate reduction; flexirubin production; hydrogen sulfide, indole and acetoin (Voges–Proskauer reaction) production; hydrolysis of agar, DNA, urea, cellulose (CM-cellulose and filter paper) and chitin; acid production from L-arabinose, D-cellobiose, L-fucose, D-lactose, D-maltose, D-melibiose, L-raffinose, L-rhamnose, L-sorbose, *N*-acetylglucosamine, adonitol, dulcitol, inositol and sorbitol; utilization of inositol, sorbitol, malonate and citrate. Data are from Nedashkovskaya *et al.* (2005) and this study.

| Characteristic | 1 | 2 | 3 |
|---|---|---|---|
| Salinity (%): | | | |
| Growth range | 0–17 | 0–15 | 0–15 |
| Optimum | 2–4 | 0–5 | 1–3 |
| Temperature (°C): | | | |
| Growth range | 10–41 | 4–37 | 4–37 |
| Optimum | 28–30 | 23–25 | 21–23 |
| Acid from: | | | |
| D-Galactose | − | + | + |
| D-Glucose | + | − | − |
| Sucrose | − | + | − |
| DL-Xylose | + | − | − |
| Mannitol | − | + | − |
| Utilization of mannitol | − | + | − |
| Susceptibility to: | | | |
| Benzylpenicillin | − | V | + |
| Tetracycline | + | + | − |
| DNA G+C content (mol%) | 42·5 | 35–36 | 38 |

**Table 2.** Whole-cell fatty acid profile of *L. blandensis* sp. nov. MED 217$^T$ compared with type strains of other *Leeuwenhoekiella* species

Strains: 1, *L. blandensis* MED 217$^T$; 2, *L. aequorea* LMG 22550$^T$; 3, *L. marinoflava* LMG 1345$^T$. Fatty acids that comprise <1 % of the total for all strains are not given.

| Fatty acid | 1 | 2 | 3 |
|---|---|---|---|
| n-15 : 0 | 9·4 | 8·1 | 11·6 |
| iso 15 : 0 | 19·4 | 16·4 | 17·1 |
| anteiso 15 : 0 | 3·5 | 0·9 | 1·2 |
| 15 : 1$\omega$6*c* | 1·2 | 1·1 | 0·8 |
| iso 15 : 1$\omega$10*c* | 13·9 | 10·9 | 12·4 |
| n-16 : 0 | 1·9 | 0·6 | 1·3 |
| iso 16 : 0 | 1·1 | 1·3 | 0·7 |
| 16 : 1$\omega$7*c* | 11·7 | 13·2 | 10·5 |
| iso 16 : 1$\omega$6*c* | 1·8 | 2·2 | 1·5 |
| iso 17 : 1$\omega$7*c* | 3·2 | 2·4 | 1·9 |
| 3-OH iso 15 : 0 | 2·5 | 1·9 | 3·1 |
| 3-OH 15 : 0 | 3·0 | 1·9 | 2·4 |
| 3-OH iso 16 : 0 | 4·1 | 6·2 | 7·2 |
| 3-OH iso 17 : 0 | 12·8 | 21·4 | 17·4 |
| 3-OH anteiso 17 : 0 | 4·7 | 4·8 | 7·1 |
| 3-OH 17 : 0 | 0·7 | 1·2 | 0·5 |

*Leeuwenhoekiella*. Therefore, we consider MED 217$^T$ to represent a novel species, for which the name *Leeuwenhoekiella blandensis* sp. nov. is proposed.

## Description of *Leeuwenhoekiella blandensis* sp. nov.

*Leeuwenhoekiella blandensis* (blan.den′sis. L. fem. adj. *blandensis* pertaining to Blande or Blanda, the name the Romans used for the city of Blanes, which has given its name to the Bay of Blanes, where the type strain was isolated).

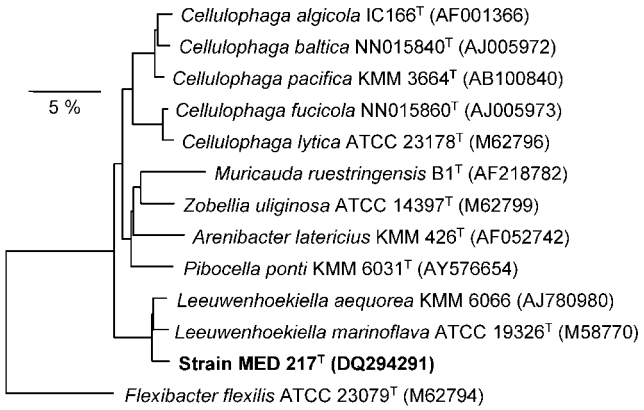


**Fig. 2.** Phylogenetic tree of representative members of the family *Flavobacteriaceae* based on nearly complete 16S rRNA gene sequences, showing the position of strain MED 217$^T$. The tree is based on the maximum-likelihood and neighbour-joining algorithms. *Flexibacter flexilis* ATCC 23079$^T$ was used as the outgroup. Bar, 0·05 substitutions per nucleotide position.

Characteristics are as given for the genus by Nedashkovskaya *et al.* (2005). In addition, cells range from 0·4 to 0·7 μm in diameter and from 1·5 to 4 μm in length. On marine agar colonies are round, 2–3 mm in diameter and yellow-pigmented with a shiny surface. Growth is observed at 10–41 °C. Optimal temperature for growth is 28–30 °C. Growth occurs at 0–17 % NaCl, with optimal growth at 2–4 % NaCl. β-Galactosidase-positive. Nitrate is not reduced. Indole, $H_2S$ and acetoin (Voges–Proskauer reaction) production are negative. Flexirubin-type pigments are not detected. Decomposes casein, gelatin, starch and Tweens 20, 40 and 80. Does not hydrolyse agar, DNA, urea, cellulose (CM-cellulose and filter paper) or chitin. Forms acid from D-glucose, DL-xylose and glycerol, but not from L-arabinose, D-cellobiose, L-fucose, D-galactose, D-lactose, D-maltose, D-melibiose, L-raffinose, L-rhamnose, L-sorbose, sucrose, *N*-acetylglucosamine, adonitol, dulcitol, inositol or mannitol. Utilizes L-arabinose, D-lactose, D-mannose and sucrose, but not inositol, mannitol, sorbitol, malonate or citrate. According to the API ZYM gallery (bioMérieux), it produces α- and β-galactosidases, acid and alkaline phosphatases, esterase lipase (C8), leucine and valine arylamidases, trypsin, α- and β-glucosidases, *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, esterase (C4), lipase (C14), cystine arylamidase, α-chymotrypsin, β-glucuronidase and α-mannosidase, but not naphthol-AS-BI-glucuronidase or α-fucosidase. Susceptible to lincomycin, doxycycline, erythromycin, chloramphenicol and tetracycline; resistant to benzylpenicillin, gentamicin, kanamycin, neomycin and polymyxin B. The G+C content of the DNA is 42·5 mol%, a value that expands the range of G+C content of members of the genus.

The type strain, MED 217$^T$ (=CECT 7118$^T$=CCUG 51940$^T$), was isolated from a surface seawater sample enriched with inorganic phosphate, from the Bay of Blanes in the north-western Mediterranean Sea on the coast of Spain.

## Acknowledgements

We thank Jean P. Euzéby for helpful advice with Latin names, and we acknowledge José Manuel Fortuño for help with scanning electron microscopy photography. We are grateful for the constructive comments on the manuscript by two anonymous reviewers. This work was supported by the Swedish Science Council (621-2003-2692) and the European Union (EVK3-CT-2002-00078 BASICS). O. I. N. is grateful to the Presidium of the Russian Academy of Sciences for a grant ('Molecular and Cell Biology').

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