NOTE

***Gelidibacter mesophilus* sp. nov., a novel marine bacterium in the family Flavobacteriaceae**M. C. Macián,^{1,2} M. J. Pujalte,^{1,2} M. C. Márquez,³ W. Ludwig,⁴ A. Ventosa,³ E. Garay^{1,2} and K. H. Schleifer⁴Author for correspondence: M. J. Pujalte. Tel: +34 96 3983142. Fax: +34 96 3983099.
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Two Gram-negative, aerobic, heterotrophic, marine bacteria, isolated from Mediterranean sea water off the coast near Valencia (Spain), were the object of this study. These non-motile, yellow-pigmented, rod-shaped strains have been studied by means of DNA–DNA hybridization, 16S rRNA sequencing and cultural and physiological features. Phylogenetic analysis showed that both strains belong to the phylum *Cytophaga–Flavobacterium–Bacteroides*, and their closest neighbour is the psychrophilic bacterium *Gelidibacter algens*. The two strains differ from *G. algens* in their mesophilic behaviour, hydrolytic pattern and use of different carbon sources. There is 31% DNA–DNA hybridization between the proposed type strain and *G. algens*, and both isolates show 97.5% 16S rDNA similarity to *G. algens*. They represent a novel species of the genus *Gelidibacter*, for which the name *Gelidibacter mesophilus* sp. nov. is proposed, with strain 2SM29^T (= CECT 5103^T = DSM 14095^T) as the type strain.

Keywords: phylum *Cytophaga–Flavobacterium–Bacteroides*, *Flavobacteriaceae*, marine bacteria, *Gelidibacter mesophilus* sp. nov., 16S rDNA phylogeny

The phylum *Cytophaga–Flavobacterium–Bacteroides* is one of the major, though poorly understood, bacterial phylogenetic groups (Woese, 1987). Polyphasic studies of its members, including phenotypic characterization, determination of fatty acid compositions and protein patterns, genomic fingerprinting and phylogenetic characterization, have demonstrated a huge degree of diversity, and several reclassifications and descriptions of new genera have been published in recent years (Bernardet *et al.*, 1996; see also references below).

The members of the phylum *Cytophaga–Flavobacterium–Bacteroides* have been isolated from very diverse natural and artificial environments (freshwater and sea water, clinical samples, air-conditioning systems, wastewater-treatment plants etc.) and show an impressive variety of phenotypes. The ability to de-

grade different biomacromolecules and complex polymers demonstrates the practical importance of these organisms. They are also known to play an important role in the biodegradation of organic matter in sea water.

The number of marine taxa in the phylum *Cytophaga–Flavobacterium–Bacteroides* has increased considerably within the last decade. Currently, there are 14 genera in the phylum that include marine species (*Cellulophaga*, *Cyclobacterium*, *Cytophaga*, *Flammeovirga*, *Flexithrix*, *Gelidibacter*, *Lewinella*, *Marinilabilia*, *Microscilla*, *Persicobacter*, *Polaribacter*, *Psychroserpens*, *Psychroflexus* and *Salegentibacter*). Most of these genera accommodate former *Cytophaga* and *Flavobacterium* species, whereas others have been described for strains newly isolated from Antarctic environments (Bowman *et al.*, 1997, 1998; Gosink *et al.*, 1998; Johansen *et al.*, 1999; McCammon & Bowman, 2000; Nakagawa & Yamasato, 1996; Nakagawa *et al.*, 1997; Raj & Maloy, 1990; Reichenbach, 1989; Sly *et al.*, 1998). Ten of the 14 genera have been described in the last 5 years. One of them, *Gelidibacter*, was proposed by Bowman *et al.* (1997), with a single species,

Abbreviations: BM, Baumann's basal medium; MA, marine agar 2216; MB, marine broth 2216; STB, salt-tolerance broth.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA sequences of *Gelidibacter mesophilus* CECT 5103^T and CECT 5104 are AJ344133 and AJ344134.

Gelidibacter algens, for a group of novel psychrophilic bacteria isolated from Antarctic sea ice. This genus belongs to the family *Flavobacteriaceae* (Bernardet *et al.*, 1996).

In a previous study (Ortigosa *et al.*, 1994), two environmental strains, 2SM28 (= CECT 5104 = DSM 14094) and 2SM29^T (= CECT 5103^T = DSM 14095^T), were isolated from Mediterranean sea water on marine agar 2216 (MA; Difco) plates. Cultures were maintained in semi-solid MA stabbs at room temperature and as suspensions in marine broth 2216 (MB; Difco) plus 10% glycerol at -80°C . They were grown routinely at $24\text{--}26^{\circ}\text{C}$ and the media used contained, or were supplemented with, NaCl at concentrations up to 2% (w/v), except in the case of those used for salinity-range testing. The phenotypic profile of the two environmental strains was determined. Most of the phenotypic tests used have been described previously (Macián *et al.*, 2001). The range of salinity that supported growth of the strains was tested in salt-tolerance broth (STB: 10 g tryptone l^{-1} ; 3 g yeast extract l^{-1}) with 0, 2, 4, 6, 8 and 10% (w/v) NaCl. The colour-shift test with 20% (w/v) KOH was performed to detect flexirubin pigmentation (Fautz & Reichenbach, 1980).

Isolation of genomic DNA, amplification of almost full-length 16S rRNA gene fragments and sequencing of rDNA by using a LICOR automated sequencer (MWG Biotech) were performed as described by Macián *et al.* (2001). Sequences were added to the 16S rRNA sequence databases of the Technische Universität München using the program package ARB (Ludwig & Strunk, 1997). The appropriate ARB tools were used for automated sequence alignment. The alignment was checked by eye and corrected manually using the sequence editor ARB_EDIT. Phylogenetic analyses were performed by applying maximum-parsimony (full dataset of 20000 sequences, ARB, Parsimony), distance-matrix (all available sequences from members of *Cytophaga-Flavobacterium-Bacteroides* as well as selected reference sequences from other major phylogenetic groups, ARB, PHYLIP as implemented in ARB; Felsenstein, 1982) and maximum-likelihood (known selected reference sequences of *Cytophaga-Flavobacterium-Bacteroides*, fastDNAm1 as implemented in ARB; Maidak *et al.*, 1996) methods to different datasets varying with respect to the inclusion of variable sequence positions (Ludwig *et al.*, 1998). The accession numbers for the 16S rDNA gene sequences used in the study are shown in Fig. 1.

DNA from our isolates, 2SM28 and 2SM29^T, as well as that of *G. algens* ACAM 536^T, was extracted and purified using the method of Marmur (1961). The G+C content of the genomic DNA was determined from the midpoint value of the thermal denaturation profile (T_m) (Marmur & Doty, 1962), as described previously (Macián *et al.*, 2001).

The DNA of strain 2SM29^T was radioactively labelled by the multiprime system with a commercial kit (RPN

Fig. 1. Phylogenetic tree derived from maximum-parsimony analysis of the 16S rRNA gene sequences of *G. mesophilus* strains CECT 5103^T and CECT 5104 and members of other related genera of the phylum *Cytophaga-Flavobacterium-Bacteroides*. Bar, 5% estimated sequence divergence.

1601 Y; Amersham) using [$1',2',5',3'$ - ^3H]dCTP (Amersham). The mean specific activity obtained with this procedure was 8.8×10^6 c.p.m. ($\mu\text{g DNA}$)⁻¹. The labelled DNA was denatured before hybridization by heating it at 100°C for 5 min and then placing it on ice. DNA-DNA similarity was studied by using the competition procedure described by Johnson (1994). Competitor DNAs were sonicated (Braun Melsungen) at 50 W for two periods of 15 s. Membrane filters (HAHY; Millipore) containing reference DNA (approx. $25 \mu\text{g cm}^{-2}$) were placed in 5 ml screw-cap vials that contained the labelled, sheared, denatured DNA and the denatured and sheared competitor DNA. The ratio of the concentration of competitor DNA to the concentration of labelled DNA was at least 150:1. The final reaction concentrations were $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M sodium chloride plus 0.015 M sodium citrate) and 30% formamide and the final volume was 140 μl . The hybridization experiments were carried out under optimal conditions, with temperatures ranging between 49 and 50°C , which is within the limits of validity for the filter method (De Ley & Tijtgat, 1970). The vials were shaken gently for 18 h in a water bath (Grant); these procedures were done in triplicate. After hybridization, the radioactivity retained by the filters was measured with a liquid scintillation counter (Beckman) and the percentage similarity was calculated as described by Johnson (1994). At least two independent determinations were carried out for each experiment; the mean values are reported here.

Cells of the two isolates were Gram-negative, strictly aerobic and rod-shaped (about $1.5\text{--}3.5 \mu\text{m}$ long and $0.5 \mu\text{m}$ wide). The cells were non-motile on wet mounts and no flagella were detected after specific staining. The strains grew on MA as mucoid, sticky, yellow colonies that did not luminesce. They did not glide on MA, but spreading growth was observed on Baumann's basal medium (BM; Baumann & Baumann,

Table 1. Phenotypic traits that allow differentiation between *Gelidibacter mesophilus* sp. nov. and other yellow-pigmented, oxidase-negative species

Data were taken from this study and from Bowman *et al.* (1997). +, Positive; –, negative; v, variable.

Characteristic	<i>G. mesophilus</i>	<i>G. algens</i>	<i>Psychroserpens burtonensis</i>
Gliding on MA	–*	+	–
Requirement for yeast extract	+	–	+
Growth at 25 °C	+	–	–
Max. growth temperature (°C)	37	< 25	< 20
Hydrolysis of Tween 80	–	v	v
Hydrolysis of starch	+	+	–
DNase	–	+	–
Use of sole carbon sources:			
D-Galactose	+	–	–
Trehalose	+	–	–
D-Mannose	+	–	–
Maltose	+	–	–
Lactose	+	–	–
D-Mannitol	–	+	–
D-Sorbitol	–	+	–
D-Gluconate	–	+	–
Pyruvate	–	+	–
Fumarate	–	+	–
Lactate	–	+	–
DNA G + C content (mol %)	39–40	36–38	27–29

* *G. mesophilus* shows spreading growth in BM with 0.1 g yeast extract l^{–1} and any of the following carbon sources: cellobiose, sucrose, melibiose, rhamnose or raffinose.

1981) with L-rhamnose, D-raffinose, sucrose, cellobiose or melibiose, thus indicating gliding motility. The isolates were strictly halophilic, requiring Na⁺ ions for growth; they were able to grow in STB containing 2–6% NaCl, but no growth was observed at NaCl contents at or above 8%. The organisms were chemo-organotrophs that were unable to ferment sugars under anaerobic conditions [as determined on Hugh & Leifson O/F medium with half-strength artificial sea water (Baumann & Baumann, 1981)]. The isolates were catalase-positive and oxidase-negative. Nitrate was not reduced to nitrite, and further growth and gas production were not observed on Baumann's denitrification medium (Baumann & Baumann, 1981). The strains grew in MB at temperatures ranging from 4 to 30 °C, but not at 37 °C. On MA plates, the strains grew at temperatures up to 37 °C, but not at 40 °C. No growth could be observed on thiosulfate/citrate/bile/sucrose (TCBS; Oxoid) agar. The strains hydrolysed starch weakly. Gelatin was hydrolysed completely after prolonged incubation (for more than 7 days). No hydrolytic activities were detected on casein, alginate, agar, Tween 80, lecithin or DNA. The strains were negative for arginine dihydrolase, lysine and ornithine decarboxylase activities and indole production. Although the strains were originally reported to grow without the addition of growth factors (Ortigosa *et al.*, 1994), they are currently unable to grow on BM

plus sole carbon sources without the addition of yeast extract. Thus, the results reported here for nutritional screening were obtained on BM supplemented with 0.1 g yeast extract l^{–1}. The strains used the following sugars as sole carbon and energy sources: D-glucose, D-galactose, trehalose, D-mannose, maltose, cellobiose, lactose and amygdalin. Very weak growth was observed on L-rhamnose, D-raffinose, sucrose and melibiose, four of the five carbon sources that allowed swarming. Flexirubin pigments were not detected.

The G + C contents of the DNA of strains 2SM28 and 2SM29^T were respectively 38.8 and 40.3 mol %, as determined by the thermal denaturation method. These values are similar to those reported for the genus *Gelidibacter* (36–38 mol %; Bowman *et al.*, 1997).

In order to investigate the phylogeny of our isolates, comparative 16S rRNA gene sequence analysis was performed. The almost complete gene sequences of the two Mediterranean sea-water isolates were determined. Phylogenetic analysis was performed by applying three alternative treeing methods and the results were congruent with respect to the positioning of the novel isolates. The sequences of the two strains were almost identical, differing at only two positions. The comparative analysis of the sequences confirmed the affiliation of the strains to the family *Flavobacteriaceae*, with *G. algens* as the next-closest relative (Fig.

1). The overall 16S rRNA sequence similarity between *G. algens* and the two strains is 97.5%. The genus *Gelidibacter* clusters within a group in which most genera are psychrophilic. The genera *Psychroserpens*, *Psychroflexus* and *Polaribacter* have recently been described as accommodating psychrophilic isolates from Antarctic environments.

The DNA–DNA relatedness experiments showed that isolate 2SM29^T shared a mean similarity value of 91% with isolate 2SM28, thus indicating that the two isolates belong to the same genospecies. On the other hand, the DNA–DNA hybridization between *G. algens* ACAM 536^T and isolate 2SM29^T was 31%, confirming that our isolates differ from *G. algens* at the species level (Owen & Pitcher, 1985; Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

The two strains can be differentiated phenotypically from the currently described *Gelidibacter* species by the characteristics reported in Table 1. Whereas the sea-ice isolate is strictly psychrophilic, unable to grow at temperatures higher than 18 °C, the Mediterranean sea-water strains are psychrotolerant and grew at temperatures up to 30 or 37 °C, depending on the medium used. The hydrolase-activity patterns are quite different: *G. algens* differs in the production of lipase and DNase. The utilization of sole carbon sources shows that *G. algens* utilizes alcohols and organic acids as sole sources of carbon and energy, whereas our isolates mainly used sugars. In contrast with *G. algens*, our isolates did not form long filaments. *G. algens* has an oxidative metabolism with glucose, as tested in O/F medium, whereas our isolates did not grow in such medium. Cells glide on BM with carbohydrates, but never on MA, as *G. algens* does. Therefore, on the basis of our polyphasic study, it is concluded that the Mediterranean sea-water isolates belong to the genus *Gelidibacter* and can be separated from the only described species of the genus, *G. algens*, by using a combination of phenotypic characteristics. Thus, it is proposed that a novel species be recognized, *Gelidibacter mesophilus* sp. nov., to include strains 2SM28 and 2SM29^T.

Description of *Gelidibacter mesophilus* sp. nov.

Gelidibacter mesophilus [me.so.phi'lus. Gr. n. *mesos* middle; Gr. adj. *philus* loving; N.L. adj. *mesophilus* middle (temperature)-loving, mesophilic].

Cells are Gram-negative rods, 1.5–3.5 µm long by 0.5 µm wide, strictly aerobic, non-flagellated. Colonies on MA are mucoid, sticky, yellow-pigmented and not luminescent. Flexirubin pigments are absent. Strains do not glide on MA, but they do glide on BM when any of the following carbohydrates are added: L-rhamnose, D-raffinose, sucrose, cellobiose or melibiose. Catalase-positive and oxidase-negative. Negative for reduction of nitrate to nitrite. No growth occurs without addition of NaCl to the culture medium or at NaCl concentrations of 8% or more. Growth occurs at temperatures

between 4 and 37 °C. Both strains are negative for the following tests: Thornley's and Møller's arginine dihydrolase, lysine and ornithine decarboxylase and indole production. Both strains hydrolyse starch and gelatin weakly, but not casein, alginate, agar, Tween 80, lecithin or DNA. The following compounds are used as sole carbon and energy sources: D-glucose, D-galactose, trehalose, D-mannose, maltose, L-rhamnose, sucrose, melibiose, cellobiose, lactose and amygdalin. Neither of the strains utilizes the following substrates: D-ribose, L-arabinose, D-xylose, D-fructose, salicin, D-gluconate, D-glucuronate, D-galacturonate, N-acetyl D-glucosamine, saccharate, glycerate, glycerol, D-mannitol, D-sorbitol, *m*-inositol, pyruvate, citrate, aconitate, 2-oxoglutarate, succinate, fumarate, DL-malate, acetate, DL-lactate, DL-β-hydroxybutyrate, *p*-hydroxybenzoate, glycine, L-leucine, L-serine, L-threonine, L-arginine, L-tyrosine, L-glutamate, L-alanine, γ-aminobutyrate, L-ornithine, L-citrulline, sarcosine and putrescine. The DNA G+C content ranges from 38.8 to 40.3 mol% (*T_m* method). Isolated from sea water from the Mediterranean coast of Valencia (Spain). The type strain, 2SM29^T, has been deposited at the Colección Española de Cultivos Tipo (Valencia, Spain) as strain CECT 5103^T and at the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 14095^T. Strain 2SM28 (= CECT 5104 = DSM 14094) is a reference strain.

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