Euzebyella saccharophila gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae*

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Strain 7SM30^T, an aerobic marine, Gram-negative, heterotrophic and yellow- to orange-pigmented bacterium isolated from seawater from Castellón, Spain, was characterized using a polyphasic approach. Analysis of the 16S rRNA gene sequence showed that the isolate represented a novel lineage within the family *Flavobacteriaceae*. The most closely related genera were *Pseudozobellia*, *Zobellia* and *Kriegella*. Cells of strain 7SM30^T were non-motile rods that required sea salts for growth, used a wide variety of carbohydrates as sole carbon and energy sources and, unlike species of the genera *Pseudozobellia* and *Zobellia*, did not possess flexirubin-type pigment or hydrolyse agar. Strain 7SM30^T contained MK6 as the sole respiratory quinone. Phosphatidylethanolamine (PE) was the only identifiable polar lipid, although other lipids were also detected. The predominant cellular fatty acids were saturated C₁₅ and monounsaturated C₁₅. The DNA G + C content was around 40 mol%. On the basis of extensive phenotypic and phylogenetic comparative analysis, it is concluded that the new strain represents a novel genus and species, for which the name *Euzebyella saccharophila* gen. nov., sp. nov., is proposed. The type strain of the type species is 7SM30^T (=CECT 7477^T=KCTC 22655^T).

The number of genera belonging to the phylum ‘*Bacteroidetes*’ has increased considerably in recent years. The family *Flavobacteriaceae* (Reichenbach, 1989; Reichenbach, 1992; emended by Bernardet *et al.*, 1996, 2002) is one of the major phylogenetic lineages within this phylum, with almost 90 recognized genera by the end of 2009. In the present study, a novel flavobacterium is described that showed low levels of 16S rRNA gene sequence similarity with members of the genus *Zobellia* and their relatives.

Strain 7SM30^T was isolated from a seawater sample obtained from a depth of 10 m off the coast of Vinaroz, Castellón, Spain, in July 1989. The seawater was decimally diluted with sterile seawater, plated on marine agar (MA; Difco) and incubated at 20–22 °C for up to 10 days. Isolated colonies

on the plates corresponding to the highest dilutions were streaked in the same medium and conditions until pure cultures were obtained. Strains were maintained by suspending cells in marine broth (MB; Difco) supplemented with 20 % glycerol at –80 °C (Ortigosa *et al.*, 1994).

The novel strain was recovered recently as a part of a project to characterize a collection of unidentified marine isolates kept at the Department of Microbiology and Ecology, University of Valencia, Spain, and was subjected to partial 16S rRNA gene sequencing. A BLAST search of public databases indicated that the sequence of strain 7SM30^T had less than 95 % similarity to the sequence of any recognized species of bacteria, with *Pseudozobellia thermophila* as the closest relative. Significant molecular and phenotypic differences found between the new strain and its relatives supported the proposal of a new genus to accommodate strain 7SM30^T.

Phenotypic characterization of strain 7SM30^T followed previously reported methods (Macián *et al.*, 2001, 2005). For comparative purposes, the following reference strains were used: *P. thermophila* CECT 7618^T, *Kriegella aquimaris* CECT 7617^T, *Zobellia russellii* CECT 7505^T and *Zobellia uliginosa* CECT 4277^T. Strains were routinely grown in MA or MB for 48–72 h at 25–28 °C for inocula or sample

Abbreviations: ASW, artificial seawater; MCS, marine cations supplement; PE, phosphatidylethanolamine; MA, marine agar; MK, menaquinone; STA, salt tolerance agar.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 7SM30^T is FN554868.

Images of colonies of strain 7SM30^T and additional phylogenetic trees constructed using the maximum-likelihood and neighbour-joining methods are available as supplementary figures with the online version of this paper. A supplementary table detailing the phenotypic characteristics of the type strains included in the study is also available.

preparation. Unless otherwise indicated, media other than MA and MB were supplemented with half-strength artificial seawater (ASW; 400 mM NaCl, 100 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mM KCl and 20 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) or marine cations supplement (MCS, Farmer & Hickman-Brenner, 2006). Suspension media for microscopic observations or miniaturized testing were also performed in salt-supplemented solutions.

Cell morphology and gliding motility were observed on wet mounts under phase-contrast microscopy. *Z. russellii* CECT 7505^T was used as a positive control for motility.

Strain 7SM30^T was Gram-negative and lysed readily when suspended in 3% (w/v) KOH solution. It did not show swarming motility, although growth on some sole carbon and energy sources (D-fructose, trehalose) showed rhizoid margins.

When grown on MA (28 °C, 48 h), strain 7SM30^T formed regular, rounded colonies with entire edges and a yellow-orange, non-diffusible pigmentation (see Supplementary Fig. S1 in IJSEM Online). The pigment did not show the colour shift characteristic for flexirubin-type pigments when colonies were flooded with 20% KOH solution (Reichenbach, 1989; Bernardet *et al.*, 2002). The ability to grow at different temperatures was tested on MA incubated at 4 °C and 15 °C (7 days) and 28, 37, and 40 °C (48 h). The strain was able to grow at temperatures from 15 to 37 °C, but no growth was seen at 4 °C or 40 °C. Ionic requirements were tested on salt tolerance agar (STA), containing 1% peptone, 0.3% yeast extract and 1.2% purified agar. This medium was supplemented with the following salt combinations (w/v): (i) with and (ii) without 2% NaCl, (iii) with 2% NaCl and 0.9% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, (iv) with 2% NaCl and 0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, (v) with 2% NaCl, 0.9% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Strain 7SM30^T did not grow without NaCl or in STA supplemented with only NaCl or Na and Ca chlorides, but showed good growth when both NaCl and MgCl_2 were added to the medium. This indicated that the strain had a specific requirement for both sodium and magnesium, but not for calcium or potassium ions. The four reference strains were tested in parallel: *P. thermophila* showed the same ionic requirements as strain 7SM30^T, *K. aquimaris* required Na, Mg and Ca ions (but no potassium), while the two species of the genus *Zobellia* tested required Na and one of the divalent cations, either magnesium or calcium, but not potassium (see Supplementary Table S1 in IJSEM Online). The salinity range for growth was tested on MA as already reported (Macián *et al.*, 2005). Strain 7SM30^T showed good growth from 0.35% to 10% total salinity and no growth with 15% or higher salinities.

Strain 7SM30^T was oxidase- and catalase-positive, unable to ferment D-glucose, D-galactose, L-arabinose, sucrose, amygdalin, melibiose or lactose in O/F medium (with MCS) and unable to grow on MA in anaerobic conditions (GENbag anaer, bioMérieux). It did not reduce nitrate to nitrite in nitrate broth or with API 20NE strips. Other activities are

given in the species description and in Supplementary Table 1. Extracellular hydrolysis of casein, DNA, starch, alginate and Tween 80 were tested after a week of incubation on MA-based media, with negative results for Tween 80, alginate and agar hydrolysis, and a weak positive result for DNase. On casein and starch, a very faint and small halo could be seen after 7 and 4 days incubation, respectively.

The results for strain 7SM30^T in API ZYM, API 20NE, API 50 CHE and Biolog GN microplate tests, performed in duplicate, are reported in the species description.

Carbon sources used for growth were tested on Baumann's basal medium [50 mM Tris/HCl, pH 7.5; 19 mM NH_4Cl ; 0.33 mM $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$; 0.1 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3% (w/v) purified agar (Oxoid) on half-strength ASW; Baumann & Baumann, 1981]. The strain grew readily in basal medium supplemented with 0.5% yeast extract (positive control) and with several carbohydrates. The growth was mucous in the latter case. Carbon sources that supported growth are indicated at the species description.

Analyses of respiratory quinones, polar lipids, cellular fatty acids and DNA G+C mol% content were carried out by the Identification Service of the DSMZ and Dr B. J. Tindall (DSM, Braunschweig, Germany). Major cellular fatty acids were determined at the DSMZ by GLC after 48 h incubation on MA at 28 °C using a previously described method (Kämpfer & Kroppenstedt, 1996). Independently, analysis of polar lipids, fatty acids and DNA G+C mol% content was also performed at the Istituto di Chimica Biomolecolare, CNR, Pozzuoli, Italy. Polar lipids and fatty acids were determined according to Romano *et al.* (2001).

Strain 7SM30^T had an MK6 menaquinone system. Analysis of polar lipids detected PE and four unidentified lipids (one unknown phospholipid). Non-saturated C_{15} and monosaturated C_{15} were the most abundant fatty acids. The major hydroxylated fatty acid was iso- $\text{C}_{17:0}$ 3-OH, as shown in Table 1. Analysis of the DNA G+C content resulted in a value of 39.5 mol% (HPLC) or 41.2 mol% using the method described by Poli *et al.* (2009). The difference between both determined values of G+C mol% content (1.7% units) could be attributed to the different methodologies. It was concluded that strain 7SM30^T had a G+C content of around 40 mol%, which was in accordance with the descriptions of other members of the family *Flavobacteriaceae*.

The almost complete 16S rRNA gene sequence of strain 7SM30^T was obtained by direct PCR amplification. This was followed by sequencing using primers 616V (forward) and 699R (reverse) as described in Arahál *et al.* (2008) for a stretch of around 1000 nt close to the 5' end, and primers P609D (forward) and P1525R (reverse) as described in Lucena *et al.* (2010) for a segment of about 750 nt close to the 3' end. The assembled and manually corrected sequence was compared with public sequences in NCBI gene databases using the BLAST program (National Center for Biotechnology Information; <http://ncbi.nlm.nih.gov/>).

Table 1. Chemotaxonomic characterization of strain 7SM30^T and *P. thermophila*, *K. aquimaris* and species of the genus *Zobellia*

Taxa: 1, strain 7SM30^T (data from this study); 2, *P. thermophila* (Nedashkovskaya *et al.*, 2009); 3, *K. aquimaris* (Nedashkovskaya *et al.*, 2008); 4, *Zobellia* (five species) (Nedashkovskaya *et al.*, 2004, 2009). All taxa possessed MK6 as the respiratory quinone. Cellular fatty acid composition was determined according to the Microbial Identification System (Microbial ID). –, Not detectable; tr, trace amount (<1 %); ND, not determined.

Characteristic	1	2	3	4
DNA G + C mol%				
Thermal denaturation	41.2	48–49	39–41	36.1–43.4
HPLC	39.5	ND	ND	36.3–43.0
Cellular fatty acids (%)				
Hydroxy fatty acids:				
C _{15:0} iso 3-OH	2.9	5.2	3.2	4.6–8.3
C _{16:0} iso 3-OH	tr	tr	tr	–
C _{17:0} iso 3-OH	11.5	22.6	12.8	15.1–25.9
Saturated				
C _{14:0}	tr	–	tr	0–1.0
C _{15:0} iso	35.0	30.8	12.3	16.8–22.5
C _{15:0}	11.0	3.0	11.1	7.5–14.4
C _{16:0} iso	tr	–	tr	–
C _{16:0}	tr	tr	tr	0.7–2.6
Unsaturated				
C _{15:1} iso G	14.7	14.8	19.7	8.8–14.9
C _{15:1} ω6c	3.0	tr	2.5	1.1–3.2
C _{16:1} ω7c/C _{15:0} iso 2-OH	6.8	6.9	9.4	9.9–15.5
C _{17:1} ω6c	tr	–	tr	0–1.2
C _{17:1} ω9c	5.0	5.4	6.4	2.4–5.1
C _{17:1} iso I/C _{17:1} anteiso B	1.0	–	1.0	–

Related sequences were analysed further using the ARB program package (Ludwig *et al.*, 2004; <http://www.arb-home.de>). Sequence alignments were further corrected manually using the ARB_EDIT sequence editor. Phylogenetic analysis using alternative treeing methods (maximum-parsimony, maximum-likelihood and neighbour-joining) and data subsets were performed using the appropriate ARB tools (Ludwig *et al.*, 1998).

Analysis of the 16S rRNA gene sequence of strain 7SM30^T (1450 nt) revealed its phylogenetic position within the family *Flavobacteriaceae*, having *K. aquimaris* and the recently described species *P. thermophila* as the closest relatives. The sequence of strain 7SM30^T showed maximum gene sequence similarity to that of *P. thermophila* (94.8 %) followed by *K. aquimaris* (93.3 %) and three species of the genus *Zobellia*, *Z. rusellii*, *Z. galactinivorans* and *Z. uliginosa*, with similarities of 94.3, 93.1 and 92.3 %, respectively. Less than 92 % 16S rRNA gene sequence similarity was found with other species of the family. The maximum-parsimony-based phylogenetic tree is presented in Fig. 1; additional information about the robustness of the tree topology, bootstrap values and node stability was

obtained by using additional treeing methods (see Supplementary Figs S2 and S3 in IJSEM Online). Taking together the tree topology and 16S rRNA gene sequence similarities, it was concluded that strain 7SM30^T represented a new taxon, probably a novel genus, in the vicinity of the genera *Pseudozobellia*, *Kriegella* and *Zobellia*. The G + C mol% content, of around 40 mol%, was similar to the values described for the genera *Kriegella* and *Zobellia*, but was distinct from the value for the genus *Pseudozobellia* (Table 1). The quinone composition was in agreement with genera described within the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). The cellular fatty acid composition was also somewhat similar to those of the closely related genera. However, given the low 16S rRNA gene sequence similarity values and several phenotypic differences, this new taxon should be considered to represent a novel genus. The absence of flexirubin-type pigment, agarase activity and gliding motility enabled the differentiation of strain 7SM30^T from members of the genera *Pseudozobellia*, *Kriegella* and *Zobellia*. Differences in the enzymic activities, temperature range for growth, salinity ranges and ionic requirements also differentiated strain 7SM30^T from closely related species (Table 2). Thus, it is concluded that strain 7SM30^T represents a new genus and novel species for which the name *Euzebyella saccharophila* gen. nov., sp. nov. is proposed.

Description of *Euzebyella* gen. nov.

Euzebyella (Eu.ze.by'el.la. N.L. fem. n. *Euzebyella* named after the French microbiologist J. P. Euzéby, for his outstanding contribution to the nomenclature of Prokaryotes and to bacterial taxonomy in general).

Gram-negative, aerobic, mesophilic, chemo-organotrophic, slightly halophilic, pigmented bacteria. Cells are non-motile, straight rods. No growth can be observed without seawater or without the addition of marine cations to the medium. Flexirubin-type pigment is not produced. Oxidase- and catalase-positive, unable to reduce nitrates. Respiratory quinone is MK6. Predominant fatty acids are iso-C_{15:0}, iso-C_{15:1} G and the major hydroxylated fatty acid is iso-C_{17:0} 3-OH. Polar lipids include PE and other unidentified lipids. The DNA G + C content is around 40 mol%. The type species is *Euzebyella saccharophila*.

Description of *Euzebyella saccharophila* sp. nov.

Euzebyella saccharophila [sac.cha.ro'phi.la. Gr. n. *saccharon* sugar; N.L. adj. *philus* -a -um (from Gr. adj. *philos* -ê -on) friend, loving; N.L. fem. adj. *saccharophila* sugar-loving].

In addition to the characteristics given for the genus, the species displays the following traits: cells are approximately 0.4 µm in width and 1.4–5 µm in length. Growth occurs on MA as regular colonies with a non-diffusible yellow–orange pigmentation. Growth is mucous on sugar-containing media. Temperature range for growth is 15–37 °C, no growth at 4 or 40 °C. Good growth is obtained in media

Fig. 1. Maximum-parsimony phylogenetic tree based on almost complete 16S rRNA gene sequences of strain 7SM30^T and closely related species. GenBank accession numbers are given in parentheses. Bootstrap values >70% are shown at branching points (percentage of 1000 resamplings). Nodes that were also obtained in the maximum-likelihood and neighbour-joining trees are indicated by open and filled circles, respectively. Bar, 1 substitution per 100 nt positions.

with 3.5–100 g l⁻¹ marine salts, no growth occurs at 150 g l⁻¹. Requires sodium and magnesium ions for growth. Does not produce arginine dihydrolase, lysine or ornithine decarboxylases, urease or indole from tryptophan. Hydrolyses aesculin and DNA (weakly), but does not hydrolyse gelatin, casein, alginate, agar, starch or Tween 80. Does not produce acid in O/F medium (with MCS) from D-glucose, D-galactose, sucrose, L-arabinose, amygdalin or melibiose, either in aerobic or anaerobic conditions. Carbon sources that sustain abundant growth in basal medium are preferentially carbohydrates: L-arabinose, D-xylose, D-glucose, D-fructose, D-galactose, trehalose, D-mannose, L-rhamnose (weak), maltose, cellobiose, sucrose, lactose, melibiose, salicin, D-gluconate and N-acetyl-D-glucosamine. The following organic acids, amino acids and carbohydrates are not used: acetate, pyruvate, propionate, butyrate, citrate, t-aconitate, 2-oxoglutarate, succinate, fumarate, malate, lactate, 3-hydroxybutyrate, glycine, L-leucine, L-serine, L-threonine, L-glutamate, L-alanine, L-arginine, L-tyrosine, L-ornithine, L-citrulline, 4-aminobutyrate, L-aspartate, L-lysine, L-histidine, L-sarcosine, putrescine, D-ribose, amygdalin, D-glucuronate, D-galacturonate,

D-glycerol, D-mannitol, D-sorbitol, *myo*-inositol, D-glycerate and D-saccharate. The following activities with API ZYM tests are positive: alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, N-acetyl β -glucosaminidase and α -mannosidase. Esterase (C4), trypsin and naphthol-AS-BI-phosphohydrolase are weak. Lipase (C14), cystine arylamidase, α -chymotrypsin, β -glucuronidase and α -fucosidase are negative. API 20NE strips give positive responses for aesculin hydrolysis, β -galactosidase and assimilation of D-glucose, L-arabinose, N-acetylglucosamine and maltose. Gluconate and pyruvate give a weak response. Nitrate reduction, indole production, D-glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis and assimilation of D-mannitol, caprate, adipate, malate, citrate and phenylacetate are negative. The following carbohydrates are acidified in API 50 CHE tests (48 h): D-arabinose, L-arabinose, D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, methyl α -D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose and sucrose. A slight acidification is noted on D-ribose,

Table 2. Differential characteristics between strain 7SM30^T, *P. thermophila*, *K. aquimaris* and species of the genus *Zobellia*

Taxa: 1, strain 7SM30^T (this study); 2, *P. thermophila* (Nedashkovskaya *et al.*, 2009); 3, *K. aquimaris* (Nedashkovskaya *et al.*, 2008); 4, *Zobellia* (five species) (Nedashkovskaya *et al.*, 2004, 2009). +, Positive; –, negative; v, variable among species.

Characteristic	1	2	3	4
Cell size (µm)	0.4 × 1.4–5.0	0.2–0.4 × 1.0–3.2	0.4–0.5 × 1.2–1.4	0.2–0.5 × 1.2–8.0
Flexirubin-type pigment	–	+	+	+
Gliding motility	–	+	+	+
Growth at/in:				
37 °C	+	+	–	v
0.35 % salinity	+	+*	–*	+†
10 % salinity	+	–*	–*	–†
Magnesium requirement	+	+*	+*	–‡
Calcium requirement	–	–*	+*	–‡
Nitrate reduction	–	–	–	+
Hydrolysis of:				
Agar	–	+	–	+
Alginate	–	+	–	+§
Tween-80	–	–	+	–†
Gelatin	–	+	–	+
Enzyme activity:				
α-Fucosidase	–	+	–	–†

*Data obtained in this study.

†Data obtained in this study for *Z. uliginosa* CECT 4277^T and *Z. russellii* CECT 7505^T.

‡Data obtained in this study for *Z. uliginosa* CECT 4277^T and *Z. russellii* CECT 7505^T: both species require divalent cations but non-specifically since they can grow with either Ca or Mg.

§Negative for *Z. laminariae*.

methyl β-D-xylopyranoside, D-galactose, L-sorbose, methyl α-D-glucopyranoside, melibiose, trehalose, inulin, raffinose, starch and D-lyxose. A negative response is obtained with glycerol, erythritol, L-xylose, D-adonitol, dulcitol, inositol, D-mannitol, D-sorbitol, melezitose, glycogen, xylitol, gentiobiose, turanose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. Substrates oxidized in Biolog GN microplates after 48 h incubation are: α-cyclodextrin, dextrin, glycogen, Tween 40, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucosamine, D-psicose, raffinose, D-sorbitol, sucrose, trehalose, turanose, D-gluconic acid and glucose 1-phosphate. Weak responses are obtained with the following substrates: adonitol, myo-inositol, L-rhamnose, xylitol, D-galacturonic acid, α-ketoglutaric acid, succinamic acid, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, L-ornithine, inosine, uridine, DL-α-glycerol phosphate and glucose 6-phosphate. Negative responses are obtained with: Tween 80, D-arabitol, i-erythritol, L-fucose, D-mannitol, methylpyruvate, monomethyl succinate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenyl acetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid, malonic

acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl glycine, glycyl L-aspartic acid, L-histidine, hydroxy L-proline, L-leucine, L-phenylalanine, L-proline, L-pyroglyutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol and glycerol.

The type strain, 7SM30^T (=CECT 7477^T=KCTC 22655^T), was isolated from seawater from the western Mediterranean Sea. The DNA G + C % content of type strain is 39.5 mol% (HPLC) or 41.2 mol% (thermal denaturation).

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