Haliea mediterranea sp. nov., a marine gammaproteobacterium

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Strain 7SM29^T, an aerobic marine gammaproteobacterium isolated from seawater from Castellón, Spain, was characterized by classical phenotyping, chemotaxonomy and 16S rRNA gene sequence analysis. Strain 7SM29^T was found to be closely related to strains in the genus *Haliea* and to *Congregibacter litoralis* KT71^T, with which a genus-level cluster was formed within the NOR5/OM60 clade of the *Gammaproteobacteria*. Strain 7SM29^T was a short, motile rod with a tuft of three polar flagella. The strain grew on marine agar and formed pale-yellow colonies. Strain 7SM29^T required NaCl for growth, reduced nitrate to nitrite, degraded several polymers and showed a preference for organic acids and amino acids over carbohydrates as carbon and energy sources. Strain 7SM29^T contained Q-8 as the sole respiratory quinone. The DNA G + C content was 62.1 mol%. Phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine were the major polar lipids. The major cellular fatty acids were unsaturated C₁₆–C₁₈ compounds. On the basis of extensive phenotypic and phylogenetic comparative analysis, it is concluded that the strain represents a novel species of the genus *Haliea*, for which the name *Haliea mediterranea* sp. nov. is proposed. The type strain is 7SM29^T (=CECT 7447^T =DSM 21924^T).

The genus *Haliea* was described recently, with one species, *Haliea salexigens* (Urios *et al.*, 2008), to accommodate a marine strain isolated from seawater of the Mediterranean Sea; a second species, *Haliea rubra* (Urios *et al.*, 2009), was later isolated from the same habitat. These organisms belong to a branch of marine gammaproteobacteria that corresponds to the so-called NOR5 clade. This clade includes more than 500 16S rRNA gene sequences from public databases but, until recently, it has contained very few cultured and taxonomically characterized representatives (Yan *et al.*, 2009). The NOR5 clade is of particular interest because of the recent description of a member, *Congregibacter litoralis* KT71^T, that represents the first cultured photosynthetic aerobic gammaproteobacterium (Spring *et al.*, 2009). Until now, bacteriochlorophyll (Bchl) *a*-producing, strict aerobes were confined to the alphaproteobacteria, but the finding of *C. litoralis* KT71^T has extended the distribution to marine gammaproteobacteria. This facultative photoheterotroph is the nearest taxon to *H. rubra* in terms of sequence similarity.

Strain 7SM29^T was isolated from a seawater sample obtained not far from the area from which *H. salexigens* 3X/A02/235^T was isolated, at 10 m depth off the coast of

Vinaroz, Castellón, Spain, in July 1989. The seawater was decimally diluted in sterile seawater, plated on marine agar (MA; Difco) and incubated at 20–22 °C for up to 10 days. Isolated colonies on plates of the highest positive inoculum dilution were streaked on the same medium and subcultured under the same conditions until a pure culture was obtained. Strain 7SM29^T was maintained as cells suspended in marine broth (MB; Difco) and 20 % glycerol at –80 °C. Strain 7SM29^T was included in a numerical taxonomic study of several aerobic, marine, Gram-negative bacteria obtained from similar samples and clustered in an unidentified phenon of fairly unreactive strains (phenon 41; Ortigosa *et al.*, 1994). Recently, the strain has been recovered as part of a project to characterize a collection of unidentified marine isolates kept at the Department of Microbiology and Ecology, University of Valencia. Strain 7SM29^T was submitted to partial 16S rRNA gene sequencing and BLAST searches in public sequence databases indicated that the sequence of strain 7SM29^T had less than 95 % similarity to all sequences of formally described bacterial species, with *H. salexigens* as the closest recognized relative. The low sequence similarity suggested that strain 7SM29^T could represent a novel taxon; thus, we performed a complete characterization *de novo*.

Strain 7SM29^T was grown routinely on MA or in MB at 25–28 °C for 48–72 h to prepare inocula and samples. Unless otherwise stated, all media were supplemented with

Abbreviation: Bchl, bacteriochlorophyll.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 7SM29^T is FN398053.

half-strength artificial seawater (ASW; 400 mM NaCl, 100 mM MgSO₄·7H₂O, 20 mM KCl and 20 mM CaCl₂·H₂O). Suspension media for microscopic observations or miniaturized testing were also supplemented with ASW. The API 20NE, API ZYM (bioMérieux) and GN2 (Biolog) tests were performed in duplicate.

Strain 7SM29^T on MA (28 °C, 48h) formed regular, rounded, translucent colonies with entire edges and a slightly yellow, non-diffusible pigmentation. It did not show swarming motility. No luminescence was observed. The ability to grow at different temperatures was tested on MA at 4 and 15 °C (15 days) and at 28, 37, 40 and 45 °C (48 h). The strain was able to grow at 15–40 °C, but not at 4 or 45 °C. Ion requirements were tested on media containing 1% peptone, 0.3% yeast extract and 1.2% bacteriological agar with and without 2% (w/v) NaCl. Strain 7SM29^T did not grow without NaCl and grew slowly after 4 days on NaCl-supplemented medium. The range of salinity for growth was tested on MA as reported previously (Macián *et al.*, 2005). The strain showed good growth with 0.35–12% total salinity, weak and slow growth with 15% salinity and no growth with 18% salinity or higher. Strain 7SM29^T was oxidase- and catalase-positive, able to reduce nitrate to nitrite (no gas) in nitrate broth and with API 20NE strips and unable to ferment glucose on O/F

medium. Extracellular hydrolysis of casein, DNA, starch and Tween 80 was detected after 1 week on MA-based media, although the halo of hydrolysis was usually small. Gelatin was hydrolysed in MB supplemented with 12% (w/v) gelatin (liquefaction of the aerobic zone of the tube) but API 20NE gave a negative response to this test. The results with API 20NE, API ZYM and Biolog GN2 are reported in the species description and Table 1.

Cell morphology, motility and flagellar arrangement were observed on wet mounts by phase-contrast microscopy. Cell size was determined by using scanning electron microscopy at the Servicio Central de Soporte a la Investigación Experimental (University of Valencia). Samples were examined in a Hitachi S-4100 field emission scanning microscope with a 7–15 mm working distance and at an acceleration voltage of 10 kV. Pictures were stored digitally and processed using the software Quantax 200. Cells of strain 7SM29^T are small, straight, regular bacilli, 0.4–0.5 µm wide and 1.1–1.3 µm long with rounded ends, single or in pairs (Fig. 1), motile by means of one, two or three monopolar flagella, as seen after flagellar staining with the method of Heimbrook *et al.* (1989). Occasionally, the strain showed a jerky type of motility that might be considered twitching motility. The cells did not clump or form evident aggregates, although extracellular

Table 1. Differential characteristics among strains of the genera *Haliea* and *Congregibacter*

Strains: 1, *Haliea mediterranea* sp. nov. 7SM29^T (data from this study); 2, *H. salexigens* 3X/A02/235^T (Urios *et al.*, 2008); 3, *H. rubra* CM41_15a^T (Urios *et al.*, 2009); 4, *C. litoralis* KT71^T (Spring *et al.*, 2009). +, Positive; w, weak; –, negative; ND, no data available. All strains contained ubiquinone Q-8.

Characteristic	1	2	3	4
Cell morphology	Short rods	Rods	Rods	Pleomorphic
Motility/flagella	+ /3 polar	+ /1 polar	– /–	+ /1–2 subpolar
Cell dimensions (µm)	0.4–0.5 × 1.1–1.3	0.3–0.7 × 1.3–1.9	0.5 × 2.7	0.4–0.7 × 0.5–4.5
Bchl <i>a</i> production	–	ND	ND	+
Growth at/with:				
40 °C	+	–	+	–
Salts (g l ^{–1})	3.5–150	7–70	7–42	10–150
10–12% salts	+	–	–	+
Hydrolysis of casein, gelatin, starch	+	ND	ND	–
Utilization of:*				
L-Alanine	+	–	–	+
α-D-Glucose	–	–	+	–
Glycerol	–	+	–	+
L-Leucine	+	+	–	ND
Acetic acid	+	–	–	w
Propionic acid	+	–	–	w
Pyruvate	+	+	–	+
Succinate	–	+	–	+
Polar lipids†	PG, DPG, PE, 2 AL, GL	PG, DPG, AL	ND	PG, PE, PL
DNA G + C content (mol%)	62.1	61.4	64.8	57.8

*Data were taken from the Biolog GN2 system for columns 1–3 and from growth on sole carbon sources for columns 1 and 4.

†DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; GL, atypical glycolipid; PL, unidentified phospholipid.

Fig. 1. Scanning electron micrographs showing the cell morphology of strain 7SM29^T. Dividing cells can also be discerned. Bars, 500 nm (a) and 2 μm (b).

filaments and material are seen in photomicrographs of pairs and short chains of cells. Cell division is by binary fission. The strain was Gram-negative and cells lysed readily when suspended in 3% (w/v) KOH solution.

Carbon sources for growth were tested on Baumann's basal medium [BMA; 50 mM Tris/HCl (pH 7.5), 19 mM NH₄Cl, 0.33 mM K₂HPO₄·3H₂O, 0.1 mM FeSO₄·7H₂O, 1.3%, w/v, purified agar (Oxoid), half-strength ASW; Baumann & Baumann, 1981] in three independent trials. The strain grew readily on medium supplemented with 0.5% yeast extract, but grew very slowly with sole carbon sources, showing no evident growth after 7 days. Carbon sources that supported growth after 15 days of incubation are given in the species description and Table 1. The pigmentation remained pale yellow during growth with single carbon sources.

To determine the ability of strain 7SM29^T to produce Bchl *a* when grown on poor or minimal medium, cellular acetone extracts were obtained from cells grown in the dark on BMA plates containing a carbon source that supported growth (such as putrescine, acetate or propionate). The absorbance spectra of the extracts were analysed using a

Table 2. Cellular fatty acid profiles of strain 7SM29^T and its closest phylogenetic neighbours

Strains: 1, *Haliae mediterranea* sp. nov. 7SM29^T (data from this study); 2, *H. salexigens* 3X/A02/235^T (Urios *et al.*, 2008); 3, *H. rubra* CM41_15a^T (Urios *et al.*, 2009); 4, *C. litoralis* KT71^T (Spring *et al.*, 2009). Values are percentages of total fatty acids. All fatty acid methyl ester profiles correspond to those of cells grown under aerobiosis; —, not detected.

Cellular fatty acid	1	2	3	4
Hydroxy				
C _{9:0} 3-OH	—	—	—	0.5
C _{10:0} 3-OH	0.8	1.8	—	2.1
C _{11:0} 3-OH	2.0	3.3	—	0.5
iso-C _{11:0} 3-OH	—	3.3	—	0.1
C _{12:0} 3-OH	1.0	1.1	—	0.4
C _{12:0} 2-OH	—	—	1.1	—
C _{12:1} 3-OH	—	—	3.5	—
Saturated				
C _{10:0}	0.8	—	—	0.5
C _{11:0}	1.5	1.0	—	1.0
C _{12:0}	1.1	1.6	—	2.2
C _{13:0}	1.8	1.3	—	1.0
C _{14:0}	2.5	1.3	0.7	2.0
C _{15:0}	—	4.5	1.2	4.9
C _{16:0}	11.2	2.0	13.0	5.4
C _{17:0}	7.5	9.3	1.0	3.1
C _{18:0}	0.3	—	0.7	0.6
Unsaturated				
C _{15:1} ω6c	0.9	5.8	0.3	2.0
C _{16:1} ω6c	—	—	—	6.5
C _{16:1} ω7c	17.2	21.2	30.5	23.1
C _{17:1} ω6c	—	2.7	1.1	2.8
C _{17:1} ω8c	19.0	23.9	2.0	8.1
C _{18:1} ω7c	22.6	17.5	40.2	29.7

Beckman Coulter DU-800 spectrophotometer, as described by Takaichi *et al.* (1991). The absorbance spectrum of strain 7SM29^T revealed no bacteriochlorophyll peak.

Analyses of respiratory quinones and polar lipids were carried out by the Identification Service of the DSMZ and Dr B. J. Tindall (DSMZ). Strain 7SM29^T had a ubiquinone (Q-8) system and the polar lipids included phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine, two unidentified aminolipids and an atypical glycolipid. Major cellular fatty acids and the DNA G+C content were determined by the Identification Service of the DSMZ after incubation on MA at 28 °C for 48 h. The results are presented in Tables 1 and 2. Monounsaturated 16–18-carbon fatty acids were the most abundant and C_{11:0} 3-OH was the major hydroxylated fatty acid. The DNA G+C content was 62.1 mol%.

The almost-complete 16S rRNA gene sequence of the strain 7SM29^T was obtained by PCR amplification followed by direct sequencing using primers 616V (forward) and 699R (reverse), as described by Arahall *et al.* (2008) to target about

Fig. 2. Neighbour-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences from strain 7SM29^T and closely related strains. Bootstrap values (>70%) based on 1000 resamplings are shown as percentages at branch nodes. Circles indicate that corresponding nodes were recovered in trees generated with the maximum-likelihood method (filled circles) and the maximum-parsimony method (open circles). Bar, 1 substitution per 100 nucleotide positions.

1000 nt close to the 5' end and primers P609D (forward, 5'-GGMTTAGATACCCBDGTA-3') and P1525R (reverse, 5'-WAGGAGGTRATCCADCC-3'), from Invitrogen/Life Technologies, to target positions 785–802 and 1525–1541, respectively (*Escherichia coli* numbering). The manually corrected sequence was compared with public sequences in the EMBL database using the BLAST program (National Center for Biotechnology Information; <http://ncbi.nlm.nih.gov/>). Related sequences were analysed further using the ARB 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 distance matrix) and data subsets were performed using the appropriate ARB tools (Ludwig *et al.*, 1998). Fig. 2 shows the results of the phylogenetic analysis. Strain 7SM29^T always clustered in a well-defined clade with the two *Haliea* type strains and *C. litoralis* KT71^T. The sequence of strain 7SM29^T had the highest 16S rRNA gene sequence similarity to those of *H. salexigens* 3X/A02/235^T and *H. rubra* CM41^T (95.0 and 95.2 %, respectively) and to the recently described *C. litoralis* KT71^T (94.8 %). Low sequence similarities were observed between strain 7SM29^T and its next closest phylogenetic neighbours, *Spongiibacter marinus* HAL40b^T (91.2 %) and *Melitea salexigens* 5IX/A01/131^T (90.7 %). All of the other sequences included in Fig. 2 had less than 91 % similarity to that of strain 7SM29^T.

It can be concluded that strain 7SM29^T represents a novel taxon in the vicinity of the genus *Haliea*. In light of the low 16S rRNA gene sequence similarity, this novel taxon could be considered a novel species of *Haliea* or *Congregibacter* or even of a novel genus close to both. The phylogenetic analysis suggests a position for strain 7SM29^T in the genus *Haliea* and also points to the possibility of reclassifying *C. litoralis* KT71^T in the same genus. According to the chemotaxonomic and phenotypic data, the resemblance of

strain 7SM29^T to the two *Haliea* species is strong enough to consider it a member of this genus. The G + C content of strain 7SM29^T (62.1 mol%) is within the range reported for the genus *Haliea* (61.4–64.8 mol%) and the quinone composition, lipid and fatty acid profiles, enzyme activities and nutritional preferences for carbon sources are also in agreement with the genus description (Tables 1 and 2). Distinctive features of strain 7SM29^T are the temperature and salinity ranges for growth, flagella number and motility, pigmentation and the precise array of carbon sources that allow growth on minimal medium. *C. litoralis* KT71^T has the very distinctive properties of synthesizing Bchl *a* and growing photoheterotrophically at low organic nutrient concentrations that are not possessed by strain 7SM29^T.

Thus, we conclude that strain 7SM29^T represents a novel species of the genus *Haliea*, for which the name *Haliea mediterranea* sp. nov. is proposed.

Description of *Haliea mediterranea* sp. nov.

Haliea mediterranea (me.di.ter.ra'ne.a. L. fem. adj. *mediterranea* pertaining to the Mediterranean Sea).

Cells are Gram-negative, short bacilli, 0.4–0.5 µm wide and 1.1–1.3 µm long, and motile by means of one, two or three polar flagella. Grows on marine agar as regular, pale-yellow colonies. Grows at 15–40 °C, but not at 4 or 45 °C. Growth of cells requires Na⁺ and is enhanced by the addition of marine salts: good growth is obtained with 3.5–120 g marine salts l⁻¹, but no growth occurs with 180 g l⁻¹. Strictly chemoheterotrophic aerobe; no fermentative growth. No Bchl *a*. Oxidase- and catalase-positive. Reduces nitrates to nitrites but not to N₂. Does not produce indole from tryptophan, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase or urease. Hydrolyses aesculin, DNA, casein, gelatin, starch and

Tween 80, but not lecithin, alginate or agar. Carbon sources that sustain growth in BMA are preferentially organic acids and amino acids: acetate, pyruvate, propionate, butyrate, 3-hydroxybutyrate, L-alanine, L-arginine, L-aspartate, L-glutamate, L-leucine, L-lysine, L-threonine and putrescine. Carbohydrates are not generally used, although weak growth is observed with amygdalin, cellobiose, salicin, D-glucuronate, DL-lactate, L-tyrosine and 4-aminobutyrate. No growth is observed with L-arabinose, D-fructose, D-galactose, D-glucose, N-acetyl-D-glucosamine, maltose, lactose, melibiose, D-mannose, L-rhamnose, D-ribose, sucrose, trehalose, D-xylose, glycerol, *myo*-inositol, D-mannitol, D-sorbitol, citrate, fumarate, D-galacturonate, D-gluconate, D-glycerate, malate, 2-oxoglutarate, D-saccharate, succinate, *trans*-aconitate, glycine, L-citrulline, L-histidine, L-ornithine, L-sarcosine or L-serine. In API ZYM tests, positive for alkaline phosphatase, esterase lipase (C8) (weak), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl- β -glucosaminidase (weak). In API 20NE tests, positive for reduction of nitrate to nitrite, hydrolysis of aesculin and assimilation of caprate and negative for production of indole, arginine dihydrolase and urease, fermentation of glucose, hydrolysis of gelatin and ONPG and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, D-gluconate, adipate, citrate, malate and phenylacetate. In Biolog GN2 MicroPlates after 48 h, oxidizes Tweens 40 and 80, pyruvic acid methyl ester, acetic acid, α -hydroxybutyric acid, propionic acid, alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-glutamate, glycyl L-aspartic acid, glycyl L-glutamic acid, hydroxy-L-proline, L-leucine, L-proline and phenylethylamine. Polar lipids include phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. The respiratory lipoquinone is Q-8. The major cellular fatty acids are monounsaturated C₁₆–C₁₈ compounds and the major hydroxylated fatty acid is C_{11:0} 3-OH. The DNA G+C content of the type strain is 62.1 mol%.

The type strain is 7SM29^T (=CECT 7447^T =DSM 21924^T), isolated from seawater of the western Mediterranean Sea.

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