Haliea rubra sp. nov., a member of the *Gammaproteobacteria* from the Mediterranean Sea

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A novel aerobic, Gram-negative bacterium, designated strain CM41_15a^T, was isolated from the surface of coastal waters of the north-western Mediterranean Sea. Cells were non-motile straight rods that formed red colonies on marine agar medium. The G+C content of the genomic DNA was 65 mol %. Phylogenetic analysis of the 16S rRNA gene sequence placed the strain in the genus *Haliea* within the class *Gammaproteobacteria*. On the basis of the 16S rRNA gene sequence comparison and physiological and biochemical characteristics, strain CM41_15a^T represents a novel species, for which the name *Haliea rubra* sp. nov. is proposed. The type strain is CM41_15a^T (=DSM 19751^T=CIP 109758^T=MOLA 104^T).

Members of the genus *Microbulbifer*, proposed originally by González *et al.* (1997), have been isolated from various marine environments, including salt marshes, intertidal sediments and coastal waters. Major amounts of iso-15:0 and iso-17:1 ω 9c as well as ubiquinone Q-8 were defined as chemical markers for this genus (Yoon *et al.*, 2004). Two strains isolated from the north-western Mediterranean Sea were studied. On the basis of their 16S rRNA gene sequences, the strains were phylogenetically related to the genus *Microbulbifer*. Fatty acid analysis revealed that iso-15:0 and iso-17:1 ω 9c were either not present or present at very low levels in both strains. These differences, in addition to 16S rRNA gene dissimilarities and other phenotypic characteristics, led to the proposal of two novel species of two new genera, *Haliea salexigens* DSM 19537^T (Urios *et al.*, 2008a) and *Melitea salexigens* DSM 19753^T (Urios *et al.*, 2008b). Recently, another strain was isolated from seawater collected from the same area from which *H. salexigens* and *Melitea salexigens* had been isolated. The 16S rRNA gene sequence of the strain had a similarity of 94 % with *H. salexigens*, its nearest relative. In this paper, we have characterized this isolate and show that it possesses characteristics that allow the proposition of a novel species of the genus *Haliea*.

Seawater samples from a depth of 3 m were collected in February 2004 from the bay of Banyuls-sur-Mer (42° 29' N 3° 08' E). Subsamples were spread on marine agar 2216 plates (MA; Difco) and incubated at 25 °C for 2 weeks. Colonies were picked and purified after three subcultures. Among these colonies, an isolate that formed red-coloured colonies was obtained and designated strain CM41_15a^T.

Microscopic observations (AX70; Olympus) showed that cells of strain CM41_15a^T were non-motile rods, approximately 2.7±0.12 µm long and 0.5±0.05 µm wide. Cells were negatively stained for transmission electron microscopy (Raguénès *et al.*, 1997). The Ryu KOH reaction (Powers, 1995) led to immediate cell lysis that was confirmed by microscopy (AX70; Olympus). This positive reaction indicated that the strain was Gram-negative.

Strain CM41_15a^T was grown in marine broth 2216 (MB; Difco). To determine the range of salinities tolerated by the isolate, MB was prepared according to the composition provided by the manufacturer with the appropriate NaCl concentration. To determine the pH range, MES, PIPES, AMPSO or MOPS (Sigma) was added to MB to achieve the appropriate pH. Cultures were incubated at 30 °C under aerobic conditions. The methods used for the determination of growth parameters were as reported by Wery *et al.* (2001b). Growth was observed at 15–44 °C with optimum growth at 30 °C (see Supplementary Fig. S1, available in IJSEM Online). Strain CM41_15a^T grew at NaCl concentrations ranging from 7 to 42 g l⁻¹ and an optimum concentration could be defined as 35 g l⁻¹ (Supplementary Fig. S1). Growth occurred at pH 5.0–9.0, with an optimum

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CM41_15a^T is EU161717.

Graphs showing the effects of temperature, pH and salinity on the growth of strain CM41_15a^T are available as supplementary material with the online version of this paper.

at pH 8.0 (Supplementary Fig. S1). To investigate the possibility of growth under anaerobic conditions, cultures were placed in an anaerobic jar on MA plates at optimal

conditions of temperature, pH and salinity (Supplementary Fig. S1). No growth was observed after 10 days and thus the strain should be considered as being strictly aerobic.

Table 1. Characteristics that differentiate strain CM41_15a^T from related species

Strains: 1, *Microbulbifer maritimus* JCM 12187^T (data from Yoon *et al.*, 2004); 2, *Microbulbifer hydrolyticus* DSM 11525^T (González *et al.*, 1997); 3, *Microbulbifer salipaludis* KCCM 41586^T (Yoon *et al.*, 2003a); 4, *Microbulbifer elongatus* DSM 6810^T (Yoon *et al.*, 2003b); 5, *Melita salexigens* DSM 19753^T (Urios *et al.*, 2008b); 6, strain CM41_15a^T (*Haliea rubra* sp. nov.); 7, *H. salexigens* DSM 19537^T (Urios *et al.*, 2008a). All strains have a ubiquinone (Q-8) system. +, Positive; –, negative; (+), weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Sampling environment	Marine sediment	Salt marsh	Salt marsh	Sand and seawater	Seawater	Seawater	Seawater
DNA G + C content (mol%)	59.9	57.7	59	ND	57.2	65	61.4
Motility	–	–	–	+	+	–	+
Temperature range (optimum) (°C)	15–48 (37)	10–41 (37)	10–45 (37)	(25–30)	15–37 (30)	15–44 (30)	10–37 (25–30)
Salinity range (optimum) (g NaCl l ^{–1})	(2–4)–10	(6–30)–60	(2–3)–10	(2–3)	7–70 (28)	7–42 (35)	7–70 (42)
pH range (optimum)	5–(6.5–7.5)	6.5–8.5 (7.5)	5–(7–8)	ND	6–10 (8)	5–9 (8)	5–9 (8)
API ZYM tests							
Alkaline phosphatase	+	ND	+	ND	+	+	+
Leucine arylamidase	+	ND	+	ND	+	+	+
Valine arylamidase	–	ND	–	ND	–	+	+
Acid phosphatase	+	ND	+	ND	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	ND	+	ND	+	–	+
N-acetyl-β-glucosaminidase	–	ND	+	ND	(+)	–	–
Esterase (C4)	+	ND	+	ND	–	+	–
Esterase lipase (C8)	+	ND	+	ND	(+)	+	(+)
Substrates							
Arabinose	ND	ND	–	+	–	–	–
Cellobiose	+	+	+	+	(+)	–	–
Fructose	–	–	–	+	+	(+)	–
Galactose	ND	ND	–	+	–	–	–
Glucose	+	+	+	+	+	+	–
Lactose	–	–	–	+	(+)	+	–
Maltose	ND	ND	+	+	+	(+)	–
Mannose	–	–	–	+	+	(+)	(+)
Rhamnose	–	–	+	–	–	–	–
Sucrose	–	–	–	+	–	–	–
Glycerol	ND	–	ND	–	+	–	+
Acetate	+	+	ND	+	–	–	–
Aspartate	ND	–	ND	(+)	(+)	–	+
Citrate	–	–	ND	–	–	+	–
Glutamate	–	+	ND	+	(+)	–	(+)
Lactate	–	ND	ND	+	–	–	–
Propionate	ND	+	ND	+	–	–	–
Pyruvate	+	+	ND	ND	+	–	+
Succinate	+	+	ND	+	+	–	+
Alanine	ND	+	ND	(+)	–	–	–
Leucine	ND	+	ND	+	+	–	+
Proline	ND	+	ND	(+)	+	–	+
Serine	+	+	ND	–	–	–	–
Tween 80	ND	–	+	ND	+	–	+

The ability of strain CM41_15a^T to use various substrates was investigated using Biolog GN2 MicroPlates (Tang *et al.*, 1998), according to the manufacturer's instructions. Positive reactions were obtained for Tween 40, D-glucose, D-lactose, citrate and γ -hydroxybutyric acid. Weakly positive reactions were obtained for i-erythritol, D-fructose, maltose, D-mannose and L-pyroglutamate. A comparison between strain CM41_15a^T and its closest relatives is presented in Table 1.

Enzymic activities were investigated using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Positive reactions were obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and acid phosphatase (Table 1).

Analysis of the fatty acid methyl esters of strain CM41_15a^T grown in MB was performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The fatty acid composition for strain CM41_15a^T was as follows: 18:1 ω 7c (40.2 %), 16:1 ω 7c/iso-15 2-OH (30.5 %), 16:0 (13.0 %), 12:1 3-OH (3.5 %), 17:1 ω 8c (2.0 %), 15:0 (1.2 %), 17:1 ω 6c (1.1 %), 12:0 2-OH (1.1 %), 17:0 (1.0 %), 14:0 (0.7 %) and 15:1 ω 6c (0.3 %) (Table 2). Iso-15:0 and iso-17:1 ω 9c, two major fatty acids found in all *Microbulbifer* strains, were not detected in strain CM41_15a^T. The two major fatty acids of strain CM41_15a^T, 18:1 ω 7c and 16:1 ω 7c/iso-15 2-OH, were also major fatty acids of *H. salexigens* DSM 19537^T. The two major fatty acids of *Melitea salexigens*

Table 2. Cellular fatty acid compositions (%) of strain CM41_15a^T and related species

Strains: 1, *Microbulbifer maritimus* JCM 12187^T (data from Yoon *et al.*, 2004); 2, *Microbulbifer hydrolyticus* DSM 11525^T (González *et al.*, 1997); 3, *Microbulbifer salipaludis* KCCM 41586^T (Yoon *et al.*, 2003a); 4, *Microbulbifer elongatus* DSM 6810^T (Yoon *et al.*, 2003b); 5, *Melitea salexigens* DSM 19753^T (Urios *et al.*, 2008b); 6, strain CM41_15a^T (*Haliea rubra* sp. nov.); 7, *H. salexigens* DSM 19537^T (Urios *et al.*, 2008a). —, Not detected.

Fatty acid	1	2	3	4	5	6	7
10:0	1.3	1.7	2.4	1.6	—	—	—
11:0	—	—	—	—	0.7	—	1.0
12:0	—	—	—	—	0.3	—	1.6
13:0	—	—	—	—	0.4	—	1.3
14:0	1.0	1.2	2.6	0.7	0.8	0.7	1.3
15:0	1.6	1.5	1.7	0.9	5.3	1.2	4.5
16:0	8.7	11.4	16.3	7.1	3.9	13.0	2.0
17:0	1.3	2.9	2.2	2.5	13.0	1.0	9.3
18:0	—	1.6	1.4	1.2	0.5	0.7	—
17:0 cyclo	2.3	5.7	—	—	—	—	—
19:0 ω 8c	1.4	1.0	—	—	—	—	—
iso-11:0	10.0	5.7	4.8	6.5	—	—	—
iso-13:0	—	—	—	—	1.0	—	—
iso-15:0	25.9	24.4	19.4	20.7	2.0	—	—
iso-15:1	0.8	1.0	0.7	1.0	—	—	—
iso-16:0	—	—	—	0.5	—	—	—
iso-17:0	6.9	10.4	5.5	9.9	1.3	—	—
anteiso-17:0	—	—	—	0.8	—	—	—
iso-17:1 ω 9c	12.6	10.1	9.5	11.3	—	—	—
15:1 ω 6c	—	—	—	—	—	0.3	5.8
17:1 ω 6c	—	—	—	—	0.6	1.1	2.7
17:1 ω 8c	—	0.5	1.0	1.8	34.1	2.0	23.9
18:1 ω 5c	—	—	0.7	—	—	—	—
18:1 ω 7c	5.6	8.9	11.8	16.3	11.4	40.2	17.5
10:0 3-OH	1.7	1.0	1.2	1.6	1.7	—	1.8
11:0 3-OH	—	—	—	—	4.6	—	3.3
12:0 2-OH	—	—	—	—	—	1.1	—
12:0 3-OH	—	—	—	—	—	—	1.1
12:1 3-OH	—	—	—	—	0.5	3.5	—
16:0 2-OH	—	—	0.9	—	—	—	—
iso-11:0 3-OH	14.2	6.2	5.7	7.7	4.3	—	3.3
iso-17:0 3-OH	—	—	0.9	—	—	—	—
16:1 ω 7c/iso-15 2-OH	2.2	2.7	7.1	6.0	9.0	30.5	21.2

DSM 19753^T (17:0 and 17:1 ω 8c) were present in minor amounts in strain CM41_15a^T. As observed for *Microbulbifer* species, fatty acids 11:0, 12:0, 13:0, 11:0 3-OH and 12:0 3-OH were not detected in strain CM41_15a^T. In contrast, fatty acids 10:0, iso-11:0, iso-15:1, iso-17:0 and iso-15:0, which are always present in *Microbulbifer* species, but are not found in *H. salexigens* DSM 19537^T, were not found in strain CM41_15a^T. Fatty acids 17:1 ω 6c and 15:1 ω 6c were found in *H. salexigens* DSM 19537^T and strain CM41_15a^T, but not in any *Microbulbifer* strains studied.

Analysis of respiratory quinones and polar lipids were carried out by the Identification Service of the DSMZ and Dr Brian Tindall (DSMZ). Strain CM41_15a^T had a ubiquinone (Q-8) system.

Genomic DNA was extracted as described by Wery *et al.* (2001a). The DNA G+C content was determined by

means of thermal denaturation, using the method of Marmur & Doty (1962) and the conditions described by Raguénès *et al.* (1997). The G+C content of the genomic DNA of strain CM41_15a^T was 64.8 ± 0.9 mol% (SEM; 3 determinations). The 16S rRNA gene was amplified and sequenced as described by Agogué *et al.* (2005). The sequence was analysed as described by Urios *et al.* (2006). Strain CM41_15a^T was shown to be phylogenetically affiliated to the genus *Haliea* in the class *Gamma-proteobacteria* (Fig. 1). The closest relative was *H. salexigens* DSM 19537^T, with a sequence similarity of 94 %.

Strain CM41_15a^T could be distinguished from its nearest phylogenetically related strains based on differences in several phenotypic properties, as shown in Tables 1 and 2. Based on these differences, we propose that strain CM41_15a^T represents a novel species of the genus *Haliea*. Because of the red-coloured colonies of strain CM41_15a^T, the name *Haliea rubra* sp. nov. is proposed.

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain CM41_15a^T. The tree corresponds to an unrooted tree obtained using the neighbour-joining algorithm (with Kimura corrections). Bootstrap percentages (based on 1000 replications) are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

Description of *Haliea rubra* sp. nov.

Haliea rubra (ru'bra. L. fem. adj. *rubra* red).

Forms red colonies on MA. Cells are Gram-negative, non-motile rods, 2.7 ± 0.12 µm long and 0.5 ± 0.05 µm wide. Growth occurs at 15–44 °C (optimum, 30 °C), at pH 5.0–9.0 (optimum, 8.0) and at salinities in the range 7–42 g NaCl l⁻¹ (optimum, 35 g l⁻¹). Positive reactions with Biolog GN2 plates are obtained for Tween 40, D-glucose, D-lactose, citrate and γ-hydroxybutyric acid. Positive API ZYM reactions for enzyme activities are obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and acid phosphatase. Oxidase- and catalase-positive. Major fatty acids are 18:1ω7c, 16:1ω7c/iso-15 2-OH and 16:0. The DNA G+C content of the type strain is 65 mol%.

The type strain, CM41_15a^T (=DSM 19751^T=CIP 109758^T=MOLA 104^T), was isolated from surface seawater in the bay of Banyuls-sur-Mer (42° 29' N 3° 08' E).

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