Neptuniibacter caesariensis gen. nov., sp. nov., a novel marine genome-sequenced gammaproteobacterium

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A Gram-negative, slightly halophilic, strictly aerobic, motile chemoorganotrophic bacterium, strain MED92^T, was isolated from a surface water sample from the eastern Mediterranean Sea. Phylogenetic analysis based on its 16S rRNA gene sequence, retrieved from the whole-genome sequence, demonstrated that this isolate is unique, showing <93 % sequence similarity to species of the families *Oceanospirillaceae* and *Alteromonadaceae*. The polar lipid profile of the novel strain consisted of phosphatidylethanolamine, phosphatidylglycerol, an unknown aminophospholipid and diphosphatidylglycerol. Major fatty acids are 16 : 1ω7*c*/15 iso 2-OH (41.2 % relative amount), 18 : 1ω7*c* (35.9 %), 16 : 0 (16.1 %), 10 : 0 3-OH (5.0 %) and 18 : 0 (1.0 %). Preferred carbon sources are organic acids and amino acids. The DNA G+C content is 46.6 mol%. Based on a phenotypic, chemotaxonomic and phylogenetic analyses, it is proposed that this marine bacterium represents a novel genus and species, for which the name *Neptuniibacter caesariensis* gen. nov., sp. nov. is proposed. The type strain is MED92^T (=CECT 7075^T=CCUG 52065^T).

The family *Oceanospirillaceae* (Garrity *et al.*, 2005) contains motile and strictly respiratory aquatic organisms of primarily marine origin that cluster in 16S rRNA gene-based phylogenetic trees together with some marine genera currently allocated to the family *Alteromonadaceae* (e.g. the genera *Marinobacter* and *Marinomicrobium*) and in the vicinity of many other members of the class *Gammaproteobacteria*, most of them from aquatic environments.

In the present study, we describe a novel bacterium, strain MED92^T, isolated from a surface seawater sample from the eastern Mediterranean Sea (32° 80′ N 34° 93′ E) collected on 15 October 2000, offshore from the historic location of Caesarea. The sample was inoculated in 0.2 μm pore-size sterile-filtered seawater (1 part inoculum : 9 parts filtered seawater) and was incubated for 48 h at 28 °C in the dark. Strain MED92^T was one of the bacteria that were competitive in seawater cultures with low concentrations of organic carbon and nutrients (for details see Pinhassi & Berman, 2003). For strain isolation, 0.1 ml of a 100× dilution of sample water was spread onto ZoBell agar plates. After primary isolation and purification, strain MED92^T was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Neptuniibacter caesariensis* MED92^T is AY136116. The GenBank/EMBL/DDBJ accession number for the genome sequence is AAOW00000000.

Additional phylogenetic trees constructed using the maximum-parsimony and maximum-likelihood methods and scanning electron micrographs of cells of strain MED92^T are available as supplementary figures in IJSEM Online.

cultivated at room temperature on the same medium and stored at −80 °C in ZoBell medium with 25 % (v/v) glycerol.

Whole-genome sequencing was carried out by the J. Craig Venter Institute (USA) through the Gordon and Betty Moore foundation initiative in Marine Microbiology (https://research.venterinstitute.org/moore/). Genome sequencing indicated that the novel strain had a DNA G+C content of 46.6 mol%, which is markedly different from that of species of the genus *Marinobacterium* (55–57 mol%). Sequencing of the genome of strain MED92^T showed an annotated genome size of approximately 3.92 Mbp (3688 putative open reading frames). The complete 16S rRNA gene sequence of strain MED92^T was 1540 nucleotides in length. This sequence was compared with public sequences in EMBL gene databases using the BLAST program (National Center for Biotechnology Information; http://ncbi.nlm.nih.gov/). Related sequences were analysed further using the ARB program package (Ludwig *et al.*, 2004) (http://www.mikro.biologie.tu-muenchen.de). Sequence alignments were 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). In addition to the phylogenetic trees presented in Fig. 1 and Supplementary Figs S1 and S2 (available in IJSEM Online), further trees were generated by applying sequence filters based on sequence conservation profiles (not shown). Phylogenetic analysis revealed that strain MED92^T was related, however only distantly, to previously described bacteria, particularly members of the family *Oceanospirillaceae* but also to some members of the family *Alteromonadaceae* and some unclassified members of the class *Gammaproteobacteria*. Consistent clustering across different trees and nodes supported by high bootstrap values were only observed for the genera containing two or more species and the cluster formed by the genera *Oceanobacter–Thalassolituus–Oleispira*. The rest of the species, including strain MED92^T, formed separate lineages for which the

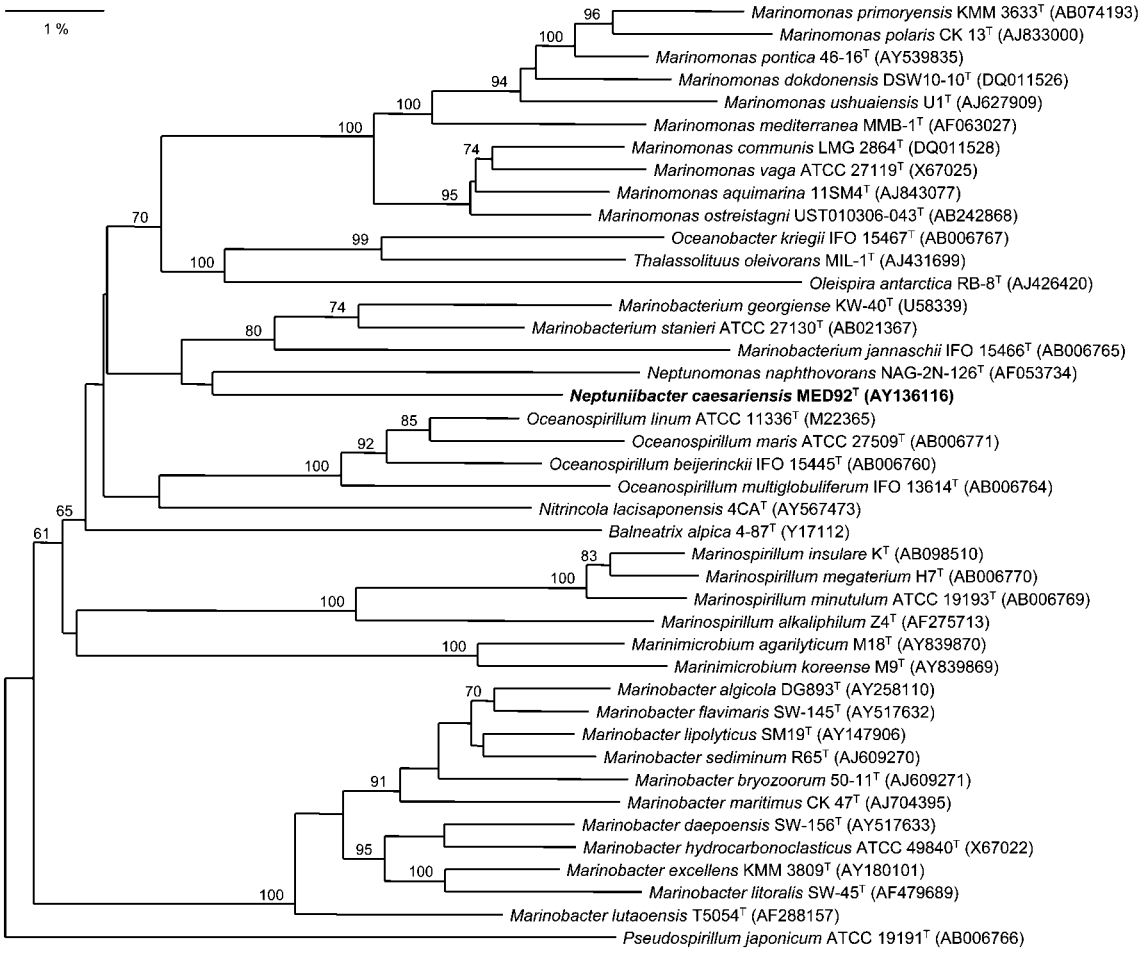

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences of *Neptuniibacter caesariensis* sp. nov. MED92^T and closely related species. Bootstrap values greater than 60 % confidence are shown at branching points (percentage of 1000 resamplings). GenBank accession numbers are given in parentheses. Bar, 1 substitution per 100 nucleotide positions.

ancestry cannot be accurately determined. In such cases, classification in separate genera seems an appropriate solution provided that other discriminating markers can be found. In terms of tree topology, the genera *Neptunomonas* and *Marinobacterium* (containing one and four species, respectively) can be considered more closely related to strain MED92^T than the rest. In terms of 16S rRNA gene sequence similarity, the highest values found were with *Oceanospirillum linum* (92.9 %), *Marinobacterium stanieri* (92.7 %), *Neptunomonas naphthovorans* (92.5 %), *Marinomonas communis* (92.3 %), *Marinobacterium georgiense* (92.2 %), *Oceanospirillum multiglobuliferum* (92.1 %) and *Marinomonas aquimarina* (92.0 %). These values are sufficiently low to consider that strain MED92^T merits classification in a separate genus. Thus, taken together, the phylogenetic analyses indicate that strain MED92^T does not belong to any of the currently recognized genera.

Strain MED92^T was subsequently investigated by using previously described methods for phenotypic characterization (Macián *et al.*, 2001, 2005). For comparative purposes, the following strains were also included in the study: *Neptunomonas naphthovorans* CECT 7132^T, *Marinobacterium georgiense* CECT 7200^T, *Marinobacterium jannaschii* CECT 7201^T, *Marinobacterium stanieri* CECT 7202^T, *Oceanospirillum linum* CECT 4190^T and *Marinomonas communis* CECT 5003^T.

Optical microscopy of bacterial cultures on wet mounts showed that cells of strain MED92^T were highly motile. For the characterization of cell morphology, cells were grown at 21 °C in marine broth 2216 (MB; Difco) until early exponential phase (24–48 h incubation), cells were then fixed with glutaraldehyde and filtered onto 0.2 μm-pore-size polycarbonate filters (Nuclepore). Samples were treated by sequential ethanol dehydration steps, critical-point drying with CO_2 and coated with silver before being viewed with a scanning electron microscope (S-3500N; Hitachi). As seen in Supplementary Fig. S3 (available in IJSEM Online), cells of strain MED92^T appear as straight, or slightly bent, rods of 0.5–0.8 μm in diameter and 1.5–2.3 μm in length; coccoid forms and short chains also occur.

Strain MED92^T grew on marine agar 2216 (MA; Difco) as regular, opaque and slightly brownish colonies that did not swarm or luminesce. Strain MED92^T required seawater-based media for growth and was unable to grow on salt tolerance agar [1 % (w/v) tryptone, 0.3 % (w/v) yeast extract and 1.5 % (w/v) agar] with the addition of Na^+ or K^+ chlorides. Negative results were also obtained when Na^+ was added together with divalent ions (Mg^{2+} or Ca^{2+}) or when all four cations were present in the medium. The salinity range supporting growth on diluted MA or in MA supplemented with NaCl, as reported in Macián *et al.* (2005), was between 1.7 and 6 % (w/v) total salts. Thus, the novel strain is a slight halophile with complex ionic requirements. Strain MED92^T is mesophilic, growing from 15 to 37 °C, but not at 4 or 40 °C on MA. All reference strains used in this study differed from strain MED92^T at the upper and lower salinity range and most of them also had a different temperature range (Table 1).

Strain MED92^T was oxidase- and catalase-positive and was unable to grow under anaerobic conditions, either through glucose fermentation or by nitrate respiration. It was negative for arginine dihydrolase, ornithine decarboxylase and indole production from tryptophan assays. No hydrolytic activities were detected on the following substrates: casein, starch, alginate, Tween 80, DNA or lecithin. These tests were performed in media supplemented with marine salts [MA or half strength artificial seawater (ASW)]. The novel strain was unable to grow on MB with 12 % (w/v) gelatin. The API ZYM gallery (bioMérieux) was used according to the manufacturer's instructions for testing the enzyme activities of strain MED92^T and the reference strains. A modification was that cells were suspended in a 35 ‰ sea salts solution before addition to the API ZYM strips and the strips were incubated for 20 h at 26 °C. Ten (out of nineteen) enzyme activities were negative for all strains tested and two activities were positive for all (although with differences in intensity). *Neptunomonas naphthovorans* CECT 7132^T and *Marinomonas communis* CECT 5003^T were the strains showing fewer and more enzyme activities (three and nine positives, respectively) on this system. The differences between the strains are shown in Table 1.

Utilization of sugars, alcohols and organic acids as sole carbon and energy sources was analysed in basal medium agar [BMA; 50 mM Tris/HCl, pH 7.5, 19 mM NH_4Cl, 0.33 mM $K_2HPO_4.3H_2O$, 0.1 mM $FeSO_4.7H_2O$ on half strength ASW solidified with 1.3 % (w/v) purified agar (Oxoid); Baumann & Baumann, 1981]. Amino acids and amines were tested as sole carbon, nitrogen and energy sources on BMA without NH_4Cl. Compounds were added at 2 g l^{-1}. Positive control plates and tubes were prepared with 5 g yeast extract l^{-1}, while negative control media consisted of BMA. Growth was monitored for 16 days. For strain MED92^T, the only compounds yielding growth were organic acids and, to a lesser extent, amino acids. Among the reference strains, *Oceanospirillum linum* CECT 4190^T and *Marinomonas communis* CECT 5003^T were the least and most versatile, respectively (Table 1). Only two strains, *Marinomonas communis* CECT 5003^T and *Neptunomonas naphthovorans* CECT 7132^T, were able to use at least some sugars and sugar alcohols. Of the three strains that grew on L-tyrosine, *Marinobacterium georgiense* CECT 7200^T and *Marinobacterium stanieri* CECT 7202^T produced a brown diffusible pigment, whereas strain MED92^T did not.

The cellular fatty acid content of strain MED92^T was determined by GLC at the DSMZ, Braunschweig, Germany, as previously described (Kämpfer & Kroppenstedt, 1996). In total, only five fatty acids with percentages above 1 % were detected. The major fatty acid was 16 : 1ω7*c*/15 iso 2-OH (41.2 %), followed by 18 : 1ω7*c* (35.9 %), 16 : 0 (16.1 %),

Table 1. Phenotypic characteristics that differentiate *Neptuniibacter caesariensis* gen. nov., sp. nov. from its closest relatives based on 16S rRNA gene sequence similarity and phylogenetic tree topology

Taxa: 1, *Neptuniibacter caesariensis* MED92^T; 2, *Neptunomonas naphthovorans* CECT 7132^T; 3, *Marinobacterium georgiense* CECT 7200^T; 4, *Marinobacterium jannaschii* CECT 7201^T; 5, *Marinobacterium stanieri* CECT 7202^T; 6, *Oceanospirillum linum* CECT 4190^T; 7, *Marinomonas communis* CECT 5003^T. +, Positive; −, negative. The scale of API ZYM values ranges from 1 (low, but detectable, activity) to 5 (high activity). A zero value (0) denotes no detectable activity. Data were obtained in this study unless otherwise indicated. Data in bold type for reference strains indicate traits not determined previously. All strains used propionate, pyruvate and lactate as a sole energy and carbon source. All strains were unable to use D-ribose, D-xylose, D-trehalose, cellobiose, lactose, *N*-acetyl-D-glucosamine or D-glycerate as a sole energy and carbon source. None of the strains showed lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase activities on the API ZYM system.

Characteristic	1	2	3	4	5	6	7
Cell morphology	Rods	Rods	Rods	Rods	Rods	Helical	Curved rods
Anaerobic growth	−	+	−	−	−	−	−
Poly-β-hydroxybutyrate	+	+	+	+	+	+	−
Salt range (%, w/v)	1.7–6.0	0.7–8.0*	0.3–8.0*	**1.4–7.0**	**0.7–15.0**	**1.4–12.0**	**0.3–15.0**
Temperature range (°C)	15–37	4–28	15–42*	15–37*	15–45*	15–37	15–42
Growth on:							
Acetate	+	+	+	+	+	−	+
L-Alanine	+	+	+	+	+	−	+
γ-Aminobutyric acid	+	**+**	**−**	+	+	**+**	+
L-Arabinose	−	+*	**−**	−	−	**−**	−
L-Arginine	−	−	−	+	−	−	+
L-Aspartate	+	**−**	+	+	−	−	+
Citrate	+	+	+	+	+	−	+
L-Citrulline	−	**−**	**−**	+	+*	−	+
D-Fructose	−	+	−*	−	−	−	+
Fumarate	+	+*	**+**	+	+	−	+
D-Galactose	−	+*	**−**	−	−	**−**	−
D-Gluconate	−	+*	**−**	−	−	**−**	+
D-Glucose	−	**+**	−*	−	−	−	+
L-Glutamate	+	+	+	+	+	−	+
Glycerol	−	+	−*	−	−	−	+
Glycine	−	−	−	+	−	−	−
L-Histidine	−	**−**	−	−	−	−	+
DL-β-Hydroxybutyrate	+	+	+	+	+	−	+
myo-Inositol	−	**+**	**−**	−	−	**−**	+
L-Leucine	−	−	−	+	−	−	−
L-Lysine	−	**−**	−*	+	−	−	+
Malate	+	+*	+	+	+	−	+
Maltose	−	−	**−**	−	−	**−**	+
D-Mannitol	−	+	**−**	−	−	**−**	+
D-Mannose	−	−	−*	−	−	**−**	+
L-Ornithine	−	−	**−**	+	+	−	+
2-Oxoglutarate	−	**+**	**+**	−	−*	−	+
Putrescine	+	**−**	**−**	+	+	−	+
L-Rhamnose	−	−	−	−	−	**−**	+
D-Saccharate	−	**+**	**−**	−	−	−	+
L-Sarcosine	−	**−**	**−**	−*	−	**−**	+
L-Serine	−	−*	−	+	−	−	+
D-Sorbitol	−	−	**−**	−	−	**−**	+
Succinate	+	+	+	+	+	−	+
Sucrose	−	−	−	−	−	−	+*
L-Threonine	−	**−**	−	+	−	−	+
L-Tyrosine	+	−	**+**	−	+	−	−
L-Tyrosine (brown pigment)	−	**−**	**+**	**−**	**+**	**−**	**−**

Table 1. cont.

Characteristic	1	2	3	4	5	6	7
Enzyme activities (API ZYM):							
Acid phosphatase	4	**4**	**1**	**1**	**5**	**0**	**5**
Alkaline phosphatase	5	**3**	**2**	**5**	**5**	**4**	**5**
Esterase (C4)	2	**0**	**1**	**2**	**2**	**3**	**2**
Esterase lipase (C8)	2	**0**	**0**	**1**	**2**	**1**	**2**
α-Glucosidase	0	**0**	**0**	**0**	**0**	**0**	**5**
β-Glucosidase	0	**0**	**0**	**0**	**0**	**0**	**1**
Leucine arylamidase	4	**3**	**4**	**4**	**3**	**2**	**4**
Naphthol-AS-BI-phosphohydrolase	3	**0**	**0**	**0**	**2**	**0**	**2**
Valine arylamidase	0	**0**	**0**	**0**	**0**	**0**	**2**
DNA G+C content (mol%)	46.6	46†	55‡	56.5§	56‖	49¶	47§

*Values for traits found in this study that differ from previously published results.
†Hedlund *et al.* (1999).
‡González *et al.* (1997).
§Baumann *et al.* (1972).
‖Baumann *et al.* (1983).
¶Bowditch *et al.* (1984).

10 : 0 3-OH (5.0 %) and 18 : 0 (1.0 %). In addition, traces (< 1 %) of the following fatty acids were also detected: 14 : 0, 20 : 1ω7*c*, and 10 : 0. According to the chemotaxonomic study of Sakane & Yokota (1994) on heterotrophic spirilla, the major fatty acids (in order of abundance) of *Oceanospirillum linum*, *Marinobacterium jannaschii* and *Oceanobacter kriegii* are 16 : 1, 18 : 1 and 16 : 0, whereas the order in *Oceanospirillum maris*, *Oceanospirillum beijerinckii*, *Oceanospirillum multiglobuliferum* and *Pseudospirillum japonicum* is 16 : 1, 16 : 0 and 18 : 1. Other combinations of the order of abundance of the same major fatty acids are found in *Marinospirillum minutulum* (16 : 0, 18 : 1, 16 : 1) (Sakane & Yokota, 1994) or *Marinobacterium stanieri*, *Marinomonas communis* and *Marinomonas aquimarina* (18 : 1, 16 : 1, 16 : 0) (Lau *et al.*, 2006). Minor constituents of the fatty acid profiles enhanced the differences between species, however a clear pattern for generic circumscription could not be found.

Analyses of polar lipids and respiratory quinones from strain MED92^T were carried out by the Identification Service of the DSMZ and by Dr B. J. Tindall, DSMZ. The results of the polar lipid analysis are presented in the species description. The major phospholipids, phosphatidylethanolamine and phosphatidylglycerol, also occur in many related marine isolates (Ivanova *et al.*, 2000, 2005; Gupta *et al.*, 2006). Analyses of quinones revealed that the novel strain has a major peak corresponding to Q8 (95 %) and two minor peaks of about the same size corresponding to unidentified components. Ubiquinone Q8 is a common characteristic among marine *Gammaproteobacteria* (Sakane & Yokota, 1994; Romanenko *et al.*, 2004).

Considering that strain MED92^T can be readily differentiated from neighbouring bacteria in different genera based on a combination of phylogenetic, genotypic, phenotypic and chemotaxonomic characteristics, we conclude that this marine bacterium represents a novel genus and species, for which the name *Neptuniibacter caesariensis* gen. nov., sp. nov. is proposed.

Description of *Neptuniibacter* gen. nov.

Neptuniibacter (Nep.tu.ni.i.bac′ter. L. adj. *Neptunius* Neptunian, pertaining to Neptune, Roman god of the sea; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Neptuniibacter* a Neptunian rod, referring to the habitat of the bacteria).

Gram-negative, strictly aerobic, chemoorganotrophic bacteria. Oxidase- and catalase-positive. Cells are rod-shaped and motile. Gas vesicles not observed. Produce poly-β-hydroxybutyrate granules. Slightly halophilic; no growth can be obtained without seawater or the addition of combined marine salts to the medium. Mesophilic. Do not ferment carbohydrates. Preferred carbon sources are organic acids and amino acids. Possess ubiquinone Q8 as a respiratory quinone. DNA G+C content is around 47 mol%. The genus is affiliated to the Class *Gammaproteobacteria*. The type species is *Neptuniibacter caesariensis*.

Description of *Neptuniibacter caesariensis* sp. nov.

Neptuniibacter caesariensis (ca.e.sa.ri.en′sis. L. masc. adj. *caesariensis* pertaining to Caesaria, as the isolate was found close to the Roman city Caesaria maritima, south of Haifa in present day Israel).

Has the following characteristics in addition to those given in the genus description. Cells are straight or slightly bent rods. Cells range from 0.5–0.8 μm in diameter and from

1.5–2.3 μm in length. Vigorous motility can be observed on fresh mounts. Does not ferment carbohydrates. Does not reduce nitrate to nitrite or gas. Requires at least 1.7 % (w/v) marine salts and tolerates up to 6 % (w/v) salts, failing to grow at 7 %. Positive growth at temperatures from 15 to 37 °C. No growth detected at 4 or 40 °C. Does not hydrolyse casein, gelatin (no growth in medium), starch, lecithin, alginate, agar, Tween-80 or DNA. Negative for arginine dihydrolase, ornithine decarboxylase and indole production from tryptophan. According to the API ZYM gallery (bioMérieux), produces acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), valine and cystine arylamidases, trypsin, α-chymotrypsin, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Utilizes the following compounds as a carbon and energy source: propionate, pyruvate, citrate, succinate, butyrate, fumarate, malate, acetate, lactate, DL-β-hydroxybutyrate, L-tyrosine (without production of a brown water-soluble pigment), L-glutamate, L-alanine, γ-aminobutyric acid, L-aspartate and putrescine. Growth is negative on: D-ribose, L-arabinose, D-xylose, D-glucose, D-fructose, D-trehalose, D-galactose, D-mannose, L-rhamnose, maltose, cellobiose, sucrose, lactose, melibiose, amygdalin, salicin, D-gluconate, D-saccharate, D-glycerate, 2-oxoglutarate, *trans*-aconitate, *N*-acetyl-D-glucosamine, glycerol, D-mannitol, *myo*-inositol, D-sorbitol, glycine, L-leucine, L-serine, L-threonine, L-arginine, L-ornithine, L-citrulline, L-histidine, L-lysine and L-sarcosine. Cellular fatty acids are, in order of abundance: 16 : 1ω7*c*/15 iso 2-OH, 18 : 1ω7*c*, 16 : 0, 10 : 0 3-OH and 18 : 0. The polar lipid profile consists of the major compounds phosphatidylethanolamine and phosphatidylglycerol, a moderate amount of an unknown aminophospholipid and a minor amount of diphosphatidylglycerol. The DNA G+C content of the type strain is 46.6 mol%.

The type strain, MED92^T (=CECT 7075^T=CCUG 52065^T), was isolated from Mediterranean Sea surface water.

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