Marinomonas blandensis sp. nov., a novel marine gammaproteobacterium

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A novel Gram-staining-negative, chemoorganotrophic, moderately halophilic, strictly aerobic bacterium, strain MED121^T, was isolated from a seawater sample collected at the Blanes Bay Microbial Observatory in the north-western Mediterranean Sea. Analysis of its 16S rRNA gene sequence, retrieved from the whole-genome sequence, showed that this bacterium was most closely related to *Marinomonas dokdonensis* and other *Marinomonas* species (96.3 and 93.3–95.7 % sequence similarities, respectively), within the family *Oceanospirillaceae*. Strain MED121^T was included into a whole-genome sequencing study and, subsequently, it was characterized using a polyphasic taxonomic approach. It was found to be oxidase and catalase positive, its cells are cocci to short rods, it does not ferment carbohydrates and does not reduce nitrate to nitrite or gas and it requires at least 2.5 % (w/v) marine salts and tolerates up to 7 % (w/v) salts. Its major cellular fatty acids in order of abundance are $C_{16:1}\omega7c$/$C_{16:1}\omega6c$, $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{10:0}$ 3-OH. Its genome had an approximate length of 5.1 million bases and a DNA G+C content equal to 40.9 mol%. Analysis of the annotated genes reveals the capacity for the synthesis of ubiquinone 8 (Q8) and the polar lipids phosphatidylglycerol and phosphatidylethanolamine, in agreement with other members of the genus. All the data collected supported the creation of a novel species to accommodate this bacterium, for which the name *Marinomonas blandensis* sp. nov. is proposed. The type strain is MED121^T (=CECT 7076^T=LMG 29722^T).

Gammaproteobacteria is one of the dominant groups of marine bacterioplankton, together with *Alphaproteobacteria* and *Bacteroidetes*, as detected by molecular biology techniques (Giovannoni & Rappé, 2000). In Blanes Bay, north-western Mediterranean Sea, *Gammaproteobacteria* account for up to 8 % (and occasionally 50 %) of the total bacterial community as shown by catalysed reporter deposition fluorescence *in situ* hybridization (Alonso-Sáez *et al.*, 2007). Phylogenetic analysis of cultured isolates and phylotypes detected by molecular biology techniques further revealed that *Gammaproteobacteria* in Blanes Bay consist of a diverse array of bacteria belonging to genera such as *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, *Marinomonas* and *Marinobacter*, as well as the SAR86 and NOR5 clades (Alonso-Sáez *et al.*, 2007; Lekunberri *et al.*, 2014). As a result of the effort to characterize the culture collection of marine bacteria isolated at the Blanes Bay Microbial Observatory (Arahal *et al.*, 2007; Pinhassi *et al.*, 2007, 2009), a number of novel *Gammaproteobacteria* were also found. One among them, strain *Marinomonas* sp. MED121^T, was subsequently selected for complete sequencing of its genome as part of a survey of phylogenetically and phenotypically

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and whole-genome sequences of *Marinomonas blandensis* MED121^T are DQ403809 and AANE00000000, respectively.

distinct bacteria thriving in the marine environment. The genus *Marinomonas*, a member of the family *Oceanospirillaceae*, contains above 20 species, distributed worldwide in marine environments, including temperate, polar and subpolar regions in both hemispheres. *Marinomonas* species are strictly aerobic chemoorganotrophs and motile by polar flagella and most of them require salt for growth.

In the present study, we describe a novel bacterium, strain MED121^T, isolated from a surface seawater sample from the Blanes Bay Microbial Observatory in the north-western Mediterranean Sea (41° 40′ N 2° 48′ E) collected on 20 March 2001. The sample was enriched with 0.6 µM P (final concentration, added as ATP), and was incubated for 72 h at 15 °C in the dark. For strain isolation, 0.1 ml of a 100× dilution of sample water was spread onto ZoBell agar plates prepared from seawater from the Bay of Blanes (indicating an abundance of approximately 1×10^3 c.f.u. ml^{-1}). Strain MED121^T was one of the bacteria that exhibited an active growth response to ATP enrichment in this seawater where bacterial growth is typically limited by the availability of P. After primary isolation and purification, strain MED121^T was cultivated at room temperature on the same medium and stored at −80 °C in ZoBell's medium with 25 % (v/v) glycerol.

The complete 16S rRNA gene sequence of strain MED121^T was 1539 nucleotides in length. This sequence was compared with corresponding sequences of the type strains within the *Marinomonas* clade using alignments retrieved from SILVA and LTP (Yarza *et al.*, 2010) latest updates as references.

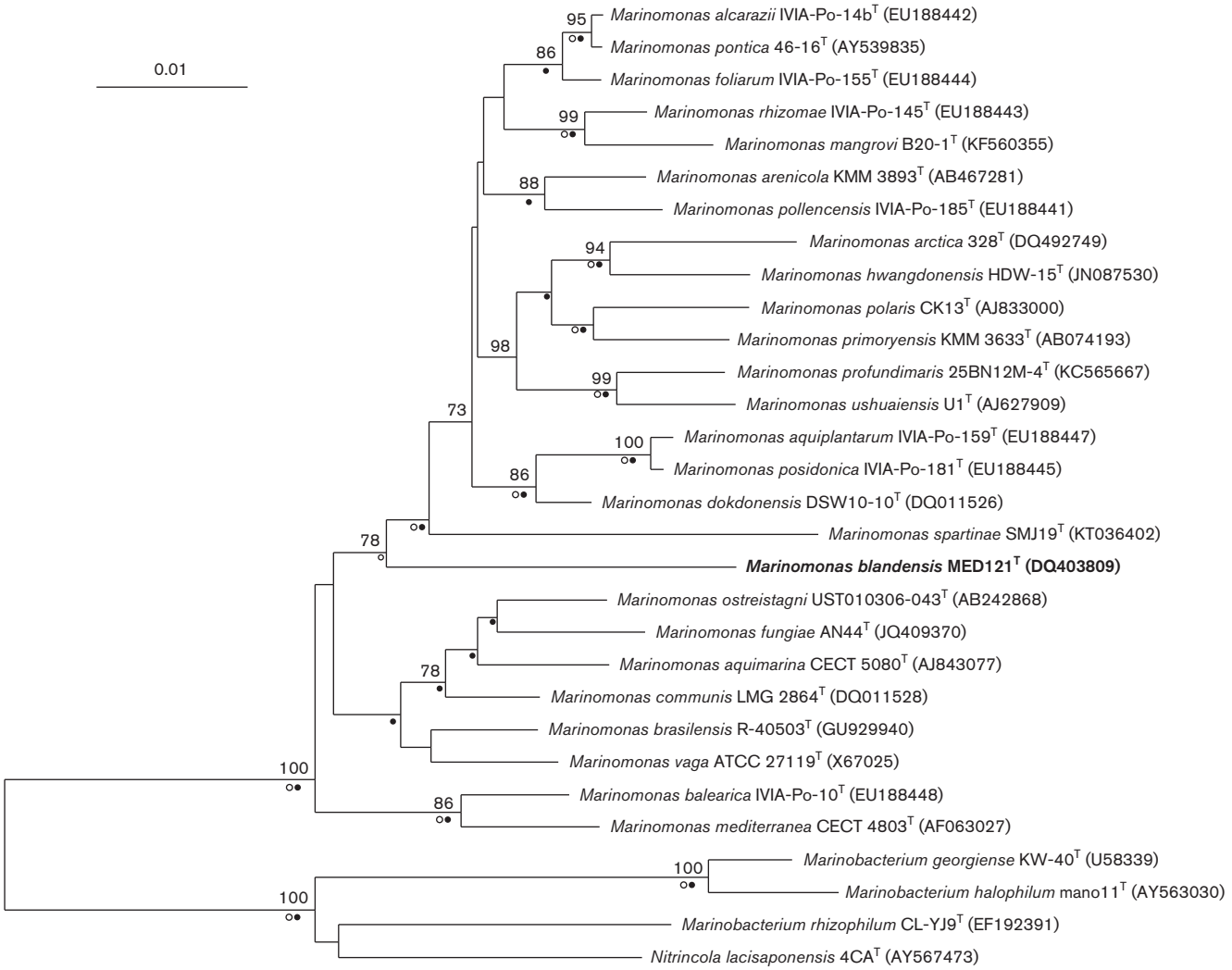

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete sequences of the 16S rRNA gene of *Marinomonas blandensis* strain MED121^T plus the type strains of other 25 *Marinomonas* species. *Marinobacterium georgiense*, *Marinobacterium halophilum*, *Marinobacterium rhizophilum* and *Nitrincola lacisaponensis* type strain sequences were added as outgroup. Sequence accession numbers are given in parentheses. Bootstrap values greater than 70 % confidence are shown at branching points (percentage of 1000 resamplings). Nodes that are obtained in the maximum-parsimony and maximum-likelihood trees are indicated by an empty circle and a filled circle, respectively. Bar, 0.01 substitutions per nucleotide position.

When necessary, additional sequences were retrieved from the GenBank/EMBL/DDBJ databases. Alignments were corrected manually based on secondary structure information. Sequence similarities were calculated in ARB based on sequence similarities without the use of an evolutionary substitution model. A phylogenetic analysis using alternative treeing methods (maximum-parsimony, maximum-likelihood and distance matrix) and data subsets was performed using the appropriate ARB tools (Ludwig *et al.*, 2004).

The highest 16S rRNA gene sequence similarities to strain MED121^T corresponded to *Marinomonas dokdonensis* DSW10-10^T, *Marinomonas brasilensis* R-40503^T and *Marinomonas posidonica* IVIA-Po-181^T with 96.3, 95.7 and 95.6 % pairwise similarity, respectively. These values are low enough to prove the taxonomic novelty of strain MED121^T and the phylogenetic tree confirms its association as an independent lineage within the genus *Marinomonas* (Fig. 1).

For routine cultivation, either Marine Agar or Marine Broth (Difco) at 26 °C, 24–48 h, has been used. The type strains of the following other *Marinomonas* species were included for comparison and characterized in parallel: *Marinomonas alcarazii* CECT 7730^T, *Marinomonas aquimarina* CECT 5080^T, *Marinomonas aquiplantarum* CECT 7732^T, *Marinomonas balearica* CECT 7378^T, *Marinomonas communis* CECT 5003^T, *Marinomonas foliarum* CECT 7731^T, *Marinomonas mediterranea* CECT 4803^T, *Marinomonas pollencensis* CECT 7375^T, *Marinomonas posidonica* CECT 7376^T, *Marinomonas rhizomae* CECT 7377^T, *Marinomonas spartinae* CECT 8886^T and *Marinomonas vaga* CECT 5004^T.

For details on cell morphology and size, cells were grown at 21 °C in Marine Broth (Difco) until early logarithmic phase (24–48 h incubation), when cells were fixed with glutaraldehyde and filtered onto 0.2 μm pore size polycarbonate filters (Nuclepore) pretreated with a drop of poly-L-lysine (0.1 % solution). Samples were treated by sequential ethanol dehydration steps, critical point drying with CO_2 and silver coating and viewed with a HITACHI S-3500N scanning electron microscope. As seen in Fig. 2, cells of strain MED121^T appear as cocci to rods, about 1.0 μm in diameter and 1.0–2.0 μm in length. Colony morphology and pigmentation were determined on Marine Agar incubated at 26 °C for 48 h.

Methods to determine temperature and salinity range, ionic requirements, oxidase and catalase activities, ability to hydrolyse casein, gelatin, starch, alginate, Tween 80 and DNA and growth with sole carbon and energy sources have been described previously (Macián *et al.*, 2005). Other tests (nitrate reduction to nitrite or N_2, indole from tryptophan, aesculin and gelatin hydrolysis, β-galactosidase activity, urease, glucose fermentation and arginine dihydrolase) were performed with API 20NE strips (bioMérieux) which were inoculated with cell suspensions on Marine Cation Supplement 1558 amended fluids (Farmer III & Hickman-Brenner, 1992). API ZYM (bioMérieux) gallery was used for testing of enzyme activities of the strain according to the manufacturer's instructions except for the inoculation as mentioned above. All tests were determined in duplicate.

Strain MED121^T is aerobic, oxidase and catalase positive. It 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^+ chloride. However, growth was 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 Marine Agar or in Marine Agar supplemented with NaCl as reported in Macián *et al.* (2005) was between 2.5 and 7 % (w/v) total salts. Thus, it is a slight halophile with complex ionic requirements. Other phenotypic features are given in Table 1 and in the species description.

Utilization of sugars, alcohols and organic acids as sole carbon and energy sources was analysed in Basal Medium Agar [50 mM Tris/HCl (pH 7.5), 19 mM NH_4Cl, 0.33 mM $K_2HPO_4.3H_2O$ and 0.1 mM $FeSO_4.7H_2O$ on half-strength Artificial Seawater (ASW) solidified with 1.3 % (w/v) Purified Agar (Oxoid); Baumann & Baumann (1981)]. Compounds were added at 2 g l^{-1}. Positive control plates were prepared with 5 g l^{-1} yeast extract while negative control media consisted of Basal Medium Agar (BMA). Growth was monitored for 12 days. Strain MED121^T was the only one in the assay that gave negative to all sources (except for the positive control plate) (Table 1).

Cellular fatty acid composition, according to standard protocols as described for the MIDI Microbial Identification System, was obtained at the CECT following previously described procedures (Sasser, 1990). The strains were cultured on Marine Agar and incubated at 26 °C for 24 h. Fatty acid methyl ester profiles of the isolates are shown in Table 2 along with the ones of the reference type strains listed above

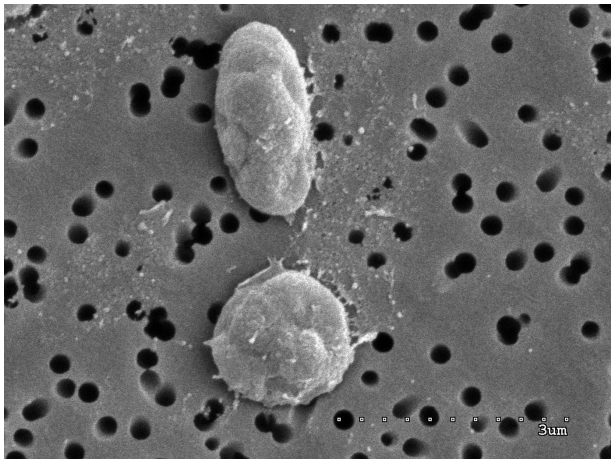

Fig. 2. Cellular morphology of *Marinomonas blandensis* MED121^T in logarithmic growth phase. Scanning electron microscopy image of cells immobilized on 0.2 μm pore size polycarbonate filter.

Table 1. Differential characteristics between *Marinomonas blandensis* and other *Marinomonas* species

Strains: 1, *Marinomonas blandensis* MED121^T; 2, *Marinomonas alcarazii* CECT 7730^T; 3, *Marinomonas aquimarina* CECT 5080^T; 4, *Marinomonas aquiplantarum* CECT 7732^T; 5, *Marinomonas communis* CECT 5003^T; 6, *Marinomonas foliarum* CECT 7731^T; 7, *Marinomonas mediterranea* CECT 4803^T; 8, *Marinomonas pollencensis* CECT 7375^T; 9, *Marinomonas posidonica* CECT 7376^T; 10, *Marinomonas rhizomae* CECT 7377^T; 11, *Marinomonas spartinae* CECT 8886^T; 12, *Marinomonas vaga* CECT 5004^T. All results are original from this study unless indicated otherwise. All strains were positive for growth at 15–26 °C, with sodium plus magnesium plus calcium ions, at 2–6 % salinity and with yeast extract on Basal Medium Agar. None of the strains was able to grow without the addition of sodium ions or hydrolyse alginate or to grow with butyrate, D-galacturonate, lactose or L-leucine.

	1	2	3	4	5	6	7	8	9	10	11	12
Brown diffusible pigment	−	−	−	−	−	−	+	−	−	−	+	−
Growth at 4 °C	−	+	−	+	−	+	−	+	−	+	+	−
Growth at 37 °C	−	+	+	+	+	−	−	+	−	−	+	+
Growth at 40 °C	−	+	+	+	+	−	−	+	−	−	−	+
Requirement of Mg^{2+}/Ca^{2+}	+	−	−	+	−	−	+	+	+	−	−	−
Growth at 7 % salinity	+	+	+	+	+	+	−	+	+	+	+	+
Growth at 12 % salinity	+	+	+	+	+	+	−	+	−	+	+	+
Growth at 15 % salinity	−	−	+	+	−	−	−	−	−	−	+	+
Hydrolysis of:												
Casein	+	−	−	−	−	−	−	−	−	−	+	−
Starch	w	−	−	−	−	−	−	−	−	−	−	−
Tween 80	−	−	−	−	−	−	+	−	−	−	−	−
Sole carbon and energy sources:												
Acetate, L-alanine, γ-aminobutyric acid, citrate, D-fructose, fumarate, D-glucose, L-histidine, 2-ketoglutarate, malate, D-mannose, pyruvate, succinate	−	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-D-glucosamine	−	+	−	+	−	+	−	−	−	−	−	+
t-Aconitate	−	−	−	+	+	−	+	+	+	+	+	−
L-Arabinose	−	+	−	+	−	+	−	−	+	−	+	+
L-Arginine	−	+	−	−	+	+	−	+	−	+	−	+
L-Aspartate	−	−	+	−	+	+	+	+	−	+	+	+
Cellobiose	−	+	−	+	−	+	+	−	+	+	+	+
L-Citrulline	−	−	−	−	+	−	−	+	−	−	−	−
D-Galactose	−	+	−	+	−	+	−	+	+	−	+	+
D-Gluconate	−	+	+	+	+	+	−	+	−	+	−	+
D-Glucuronate	−	+	−	+	−	+	−	−	+	−	−	−
L-Glutamate, glycerol	−	+	−	+	+	+	+	+	+	+	+	+
Glycine	−	−	+	−	+	−	−	−	−	+	−	−
DL-β-Hydroxybutyrate	−	−	−	+	+	−	+	−	−	+	−	−
m-Inositol	−	+	−	+	+	−	+	−	+	+	−	+
L-Lysine	−	+	−	+	+	+	−	+	−	+	+	+
Maltose	−	+	−	+	+	+	−	−	−	+	+	+
D-Mannitol	−	+	−	+	+	+	−	+	+	+	+	+
Melibiose	−	+	−	+	−	−	−	−	−	−	−	−
L-Ornithine	−	+	−	+	+	+	+	+	−	+	−	+
Propionate	−	+	+	+	+	+	−	+	+	+	+	+
Putrescine	−	+	−	−	+	+	+	+	−	−	+	+
L-Rhamnose	−	+	−	+	+	+	−	−	+	+	−	+
D-Ribose	−	+	−	+	−	−	+	+	−	−	−	−
D-Saccharate	−	+	−	−	+	−	−	−	−	−	−	+
Salicin	−	+	−	−	−	+	−	−	+	−	+	−
L-Sarcosine	−	+	+	+	+	+	−	−	−	+	+	+
L-Serine	−	+	+	+	+	+	−	+	−	−	+	+
D-Sorbitol	−	+	−	−	+	+	−	−	−	+	−	+
Sucrose	−	+	−	−	−	+	−	−	−	+	+	−
L-Threonine	−	−	−	+	+	−	−	+	−	+	+	+
Trehalose	−	+	−	−	−	+	−	−	−	−	−	−

Table 1. cont.

	1	2	3	4	5	6	7	8	9	10	11	12
L-Tyrosine	–	–	–	–	–	–	–	–	–	+	–	–
D-Xylose	–	+	–	+	–	+	–	–	+	–	+	–

and analysed in parallel. While presenting the main fatty acids that are displayed by other *Marinomonas* species, the profile of strain MED121^T differs from them in the relative abundance of the two major fatty acids, the presence of $C_{12:0}$ (detected only in 8 of the 13 types strains assayed) and the absence of other hydroxyl fatty acids aside from $C_{10:0}$ 3-OH (occurring only in seven strains in Table 2).

Whole-genome sequencing of strain MED121^T was carried out by the J. Craig Venter Institute through the Gordon and Betty Moore foundation initiative in Marine Microbiology. It resulted in an annotated genome size of approximately 5.15 Mbp (4696 putative ORF) with a G+C content of 40.9 mol%, which is very close to the lower limit of the range given in the emended description of the genus, 41–50 mol% (Espinosa *et al.*, 2010). To date, the genome of strain MED121^T consists of 47 contigs (AANE01000001–AANE01000047) organized into 9 scaffolds (CH672429–CH672437). Annotation by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline Group gave a total of 4820 proteins.

The genome of strain MED121^T contains all genes needed for phosphatidylglycerol and phosphatidylethanolamine synthesis but lacks the cardiolipin synthase gene needed for the synthesis of diphosphatidylglycerol. These synthetic abilities are in agreement with the finding that the six *Marinomonas* species so far studied for polar lipid composition contain phosphatidylglycerol and phosphatidylethanolamine as main phospholipids while diphosphatidylglycerol is not found in any of them: *Marinomonas arctica* (Zhang *et al.*, 2008), *Marinomonas hwangdonensis* (Jung *et al.*, 2012), *Marinomonas mangrovi* (Zhang & Margesin, 2015), *Marinomonas polaris* (Gupta *et al.*, 2006), *Marinomonas pontica* (Ivanova *et al.*, 2005) and *Marinomonas profundimaris* (Bai *et al.*, 2014). Regarding the main quinone type, genomic information sustains the ability of strain MED121^T to synthetize ubiquinone 8 (Q8), based on the presence of the gene coding for octaprenyl diphosphate synthase (E 2.5.1.90), but not enzymes able to synthesize larger quinone polyisoprenoids. The same is found in the genomes of nine other *Marinomonas* species.

All data gathered support that strain MED121^T represents a novel species in the genus *Marinomonas*, well delineated by phylogenetic analysis and phenotypically differentiated from other species, as reported in Tables 1 and 2, for which we propose *Marinomonas blandensis* sp. nov.

Description of *Marinomonas blandensis* sp. nov.

Marinomonas blandensis (blan.den′sis. N.L. fem. adj. *blandensis* pertaining to *Blande* or *Blanda*, the name the Romans used

Table 2. Cellular fatty acid composition of strain MED121^T and other *Marinomonas* species

Strains: 1, *Marinomonas blandensis* MED121^T; 2, *Marinomonas alcarazii* CECT 7730^T; 3, *Marinomonas aquimarina* CECT 5080^T; 4, *Marinomonas aquiplantarum* CECT 7732^T; 5, *Marinomonas balearica* CECT 7378^T; 6, *Marinomonas communis* CECT 5003^T; 7, *Marinomonas foliarum* CECT 7731^T; 8, *Marinomonas mediterranea* CECT 4803^T; 9, *Marinomonas pollencensis* CECT 7375^T; 10, *Marinomonas posidonica* CECT 7376^T; 11, *Marinomonas rhizomae* CECT 7377^T; 12, *Marinomonas spartinae* CECT 8886^T; 13, *Marinomonas vaga* CECT 5004^T. All results are original from this study. –, Absent; TR, trace amount.

	1	2	3	4	5	6	7	8	9	10	11	12	13
$C_{10:0}$	TR	TR	TR	TR	1.4	2.2	–	TR	1.0	TR	–	–	1.8
$C_{10:0}$ 3-OH	7.2	6.9	4.4	–	18.0	9.4	4.8	10.5	6.9	8.4	5.9	11.8	9.0
$C_{12:0}$	0.6	–	2.3	–	1.2	3.9	–	–	7.4	TR	–	6.6	1.7
$C_{12:1}$ 3-OH	–	4.6	–	6.2	–	–	6.5	–	–	3.8	7.2	–	–
$C_{12:0}$ 3-OH	–	–	–	–	–	–	–	–	–	–	TR	4.8	–
$C_{14:0}$	1.0	TR	1.8	1.2	4.8	1.6	1.2	3.4	TR	2.2	TR	1.9	1.7
$C_{16:0}$	19.1	17.6	16.5	21.8	15.0	10.1	17.9	18.2	18.8	21.9	14.4	24.5	13.0
SF3*	37.3	23.4	27.4	27.9	34.6	24.8	28.3	25.4	16.2	32.5	22.4	23.3	19.6
$C_{18:0}$	3.0	3.6	1.3	4.7	–	1.3	2.7	1.4	3.8	2.5	4.6	1.0	1.6
$C_{18:1}\omega7c$	30.7	42.4	45.5	37.7	25.2	45.5	38.5	40.6	45.6	27.4	43.4	25.7	42.1
$C_{18:1}\omega6c$	–	–	–	–	–	–	–	–	–	–	–	–	9.6
$C_{19:0}$ 10-methyl	–	–	–	–	–	1.1	–	–	–	–	1.0	–	–

*Summed feature 3 comprises $C_{16:1}\omega7c/C_{16:1}\omega6c$.

for the city of Blanes, which has given its name to the Bay of Blanes, where the type strain was isolated).

They are Gram-staining-negative, strictly aerobic, chemoorganotrophic bacteria, which are oxidase and catalase positive. Cells are cocci to short rods about 1.0 μm in diameter and 1.0–2.0 μm in length. Gas vesicles are not observed. It produces vaguely coloured with greenish tinge semi-transparent colonies. It does not ferment carbohydrates. It does not reduce nitrate to nitrite or gas, requires at least 2.5 % (w/v) marine salts and tolerates up to 7 % (w/v) salts. There is a positive growth from 15 to 28 °C. No growth is detected at 4 or 37 °C. It hydrolyses casein, gelatin, starch (weakly) and DNA. It does not hydrolyse alginate or agar. It is negative for arginine dihydrolase, ornithine decarboxylase, urease, aesculin hydrolysis and indole production from tryptophan. It is positive for β-galactosidase.

It does not utilize any of the compounds tested as single carbon and energy source: acetate, *N*-acetyl-D-glucosamine, *t*-aconitate, L-alanine, γ-aminobutyric acid, L-arabinose, L-arginine, L-aspartate, butyrate, cellobiose, citrate, L-citrulline, D-fructose, fumarate, D-galactose, D-galacturonate, D-gluconate, D-glucose, D-glucuronate, L-glutamate, glycerol, glycine, L-histidine, DL-β-hydroxybutyrate, *m*-inositol, 2-ketoglutarate, lactate, lactose, L-leucine, L-lysine, malate, maltose, D-mannitol, D-mannose, melibiose, L-ornithine, propionate, putrescine, pyruvate, L-rhamnose, D-ribose, D-saccharate, salicin, L-sarcosine, L-serine, D-sorbitol, succinate, sucrose, L-threonine, trehalose, L-tyrosine and D-xylose.

It is positive in API ZYM to alkaline phosphatase and leucine arylamidase. It is negative to the other enzymatic activities of this miniaturized system.

Its major cellular fatty acids in order of abundance are $C_{16:1}\omega7c$/$C_{16:1}\omega6c$, $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{10:0}$ 3-OH. The whole pattern and relative abundance is given in Table 2.

The DNA G+C content is 40.9 mol% (draft genome sequence).

The type strain MED121^T (=CECT 7076^T=LMG 29722^T) was isolated from Mediterranean Sea surface water.

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