Reinekea blandensis sp. nov., a marine, genome-sequenced gammaproteobacterium

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A novel heterotrophic, moderately halophilic, strictly aerobic, motile bacterium 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 the single-species genera *Reinekea* and *Saccharospirillum* (95 and 94 % sequence similarity, respectively) within the class *Gammaproteobacteria*. The data from phenotypic, genotypic, chemotaxonomic and phylogenetic analyses supported the creation of a novel species of the genus *Reinekea* to accommodate this bacterium, for which the name *Reinekea blandensis* sp. nov. is proposed. The type strain is MED297[T] (=CECT 7120[T] =CCUG 52066[T]).

The *Gammaproteobacteria* constitutes one of the predominant groups within the marine bacterioplankton, together with the *Alphaproteobacteria* and *Bacteroidetes*, according to data obtained using molecular biological techniques (Giovannoni & Rappé, 2000). Furthermore, the phylogenetic diversity of cultured and uncultured gammaproteobacteria accounts for 25–44 % of the global marine bacterioplankton diversity, as revealed by analysis of 16S rRNA gene sequences reported in public databases (i.e. GenBank) (Hagström *et al.*, 2002). In Blanes Bay in the 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 biological techniques revealed that the gammaproteobacteria in Blanes Bay consist of a diverse array of bacteria belonging to genera such as *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, *Marinomonas* and *Marinobacter*, and to the SAR86 and NOR5 clades (Alonso-Sáez *et al.*, 2007; I. Lekunberri and C. Pedrós-Alió, unpublished). As a result of attempts to characterize the culture collection comprising marine bacteria isolated at the Blanes Bay Microbial Observatory (Pinhassi *et al.*, 2006; Arahal *et al.*, 2007), a number of novel gammaproteobacteria were found. One among them, strain MED297[T], was subsequently selected for complete sequencing of its genome as part of a survey of phylogenetically and phenotypically distinct bacteria thriving in the marine environment.

In the present study, we describe a novel bacterium, strain MED297[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 13 November 2001. The sample was incubated for 72 h at 17 °C in the dark. For strain isolation, 0.1 ml aliquots of a $100 \times$ dilution of sampled seawater (representing an abundance of approximately 1×10^3 c.f.u. ml^{-1}) were spread onto ZoBell agar plates. After primary isolation and purification, strain MED297^T was cultivated at room temperature on the same medium and stored at -80 °C in ZoBell's medium with 25 % (v/v) glycerol.

Whole-genome sequencing was carried out at the J. Craig Venter Institute through the Gordon and Betty Moore Foundation Initiative in Marine Microbiology (https://research.venterinstitute.org/moore/). Genome sequencing showed the DNA G+C content of strain MED297^T to be 52.4 mol%. Sequencing of the genome produced an annotated genome size of approximately 4.51 Mbp (4301 putative open reading frames). The complete 16S rRNA gene sequence of strain MED297^T was 1530 nt in length. This sequence was compared with public sequences in EMBL by using the BLAST program (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Related sequences were further analysed using the program package ARB (Ludwig *et al.*, 2004; http://www.arb-home.de/). Sequence alignments were corrected manually using the sequence editor ARB_EDIT. Phylogenetic analyses using alternative treeing methods (neighbour joining, maximum parsimony and maximum likelihood) and data subsets were performed using the appropriate ARB tools (Ludwig *et al.*, 1998). The tree derived using the neighbour-joining method with Jukes–Cantor corrections is shown in Fig. 1. Phylogenetic analysis revealed that strain MED297^T was related to the species *Reinekea marinisedimentorum* and *Saccharospirillum impatiens*. However, the low levels of sequence similarity between strain MED297^T and the type strains of these two species (95.0 and 94.0 %, respectively)

indicate that they are not related at the species level. Other genera in the *Gammaproteobacteria* are clearly more distantly related (<90 % similarity). The high bootstrap percentages (Fig. 1) and the comparison of local topologies obtained using different treeing methods (see Supplementary Figs S1 and S2, available in IJSEM Online) both confirm that strain MED297^T clusters consistently with *R. marinisedimentorum* in the first instance, and thereafter with *S. impatiens*.

Strain MED297^T was subsequently investigated using previously described methods for phenotypic characterization (Macián *et al.*, 2001, 2005). For comparative purposes, *R. marinisedimentorum* CECT 7208^T and *S. impatiens* CECT 5721^T were also included in the study.

Optical microscopy of bacterial cultures on wet mounts showed that cells were highly motile. To determine the cell morphology, strain MED297^T was grown at 21 °C in marine broth 2216 (Difco) until early exponential phase (24–48 h incubation), at which point cells were fixed with glutaraldehyde and filtered onto polycarbonate filters with a 0.2 µm pore size (Nuclepore). Samples were treated using sequential ethanol dehydration steps, critical-point drying with CO_2 and silver coating before being viewed in a scanning electron microscope (S-3500N; Hitachi). As seen in Fig. 2, cells of strain MED297^T appear as single curved rods, 0.3–0.7 µm in diameter and 1.2–2.8 µm in length. In some cells, the curvature resembles a gentle spiral. Budding can also be seen at the tips of some cells.

Strain MED297^T grew on marine agar 2216 (Difco) (MA) as regular, opaque, non-pigmented colonies that did not swarm or luminesce. Strain MED297^T required seawater-based media for growth and was unable to grow on salt tolerance agar (tryptone, 1 %, w/v; yeast extract, 0.3 %, w/v; agar, 1.5 %, w/v) with the addition of sodium chloride or potassium chloride. However, growth was obtained when
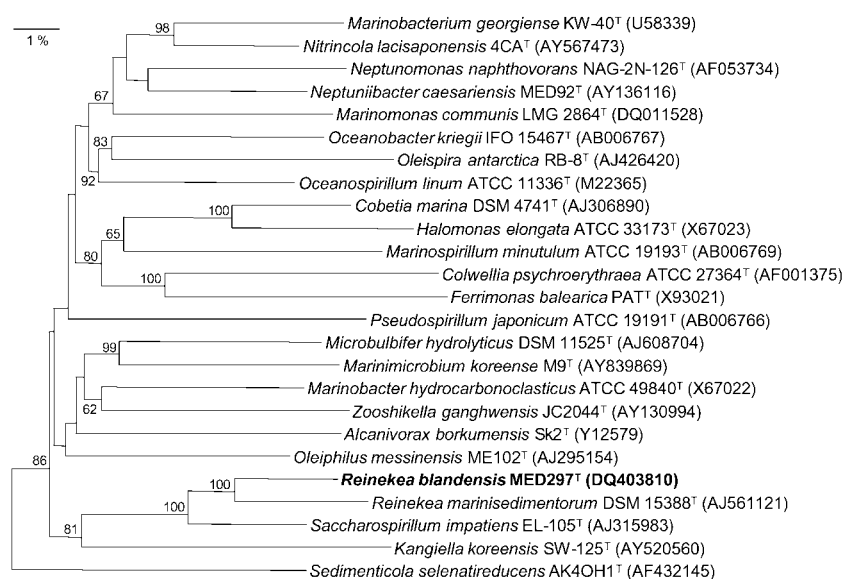

Fig. 1. Neighbour-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences of strain MED297^T and closely related species. Bootstrap percentages (based on 1000 resamplings) greater than 60 % are shown at branch points. Sequence accession numbers are given in parentheses. Bar, 1 estimated substitution per 100 nucleotide positions.
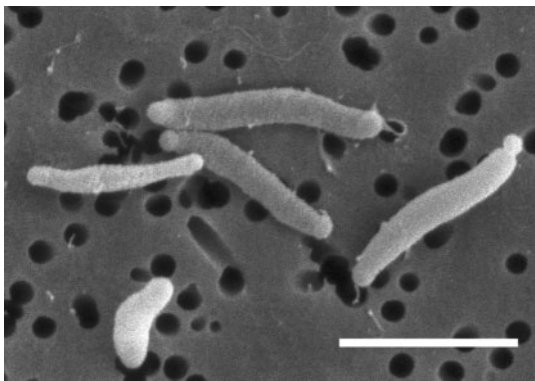
The tree includes the following taxa:

- *Marinobacterium georgiense* KW-40^T (U58339)
- *Nitrincola lacisaponensis* 4CA^T (AY567473)
- *Neptunomonas naphthovorans* NAG-2N-126^T (AF053734)
- *Neptuniibacter caesariensis* MED92^T (AY136116)
- *Marinomonas communis* LMG 2864^T (DQ011528)
- *Oceanobacter kriegii* IFO 15467^T (AB006767)
- *Oleispira antarctica* RB-8^T (AJ426420)
- *Oceanospirillum linum* ATCC 11336^T (M22365)
- *Cobetia marina* DSM 4741^T (AJ306890)
- *Halomonas elongata* ATCC 33173^T (X67023)
- *Marinospirillum minutulum* ATCC 19193^T (AB006769)
- *Colwellia psychroerythraea* ATCC 27364^T (AF001375)
- *Ferrimonas balearica* PAT^T (X93021)
- *Pseudospirillum japonicum* ATCC 19191^T (AB006766)
- *Microbulbifer hydrolyticus* DSM 11525^T (AJ608704)
- *Marinimicrobium koreense* M9^T (AY839869)
- *Marinobacter hydrocarbonoclasticus* ATCC 49840^T (X67022)
- *Zooshikella ganghwensis* JC2044^T (AY130994)
- *Alcanivorax borkumensis* Sk2^T (Y12579)
- *Oleiphilus messinensis* ME102^T (AJ295154)
- **Reinekea blandensis** MED297^T (DQ403810)
- *Reinekea marinisedimentorum* DSM 15388^T (AJ561121)
- *Saccharospirillum impatiens* EL-105^T (AJ315983)
- *Kangiella koreensis* SW-125^T (AY520560)
- *Sedimenticola selenatireducens* AK4OH1^T (AF432145)

Fig. 2. Cellular morphology of strain MED297^T in exponential growth phase. Scanning electron microscopy images of cells immobilized on polycarbonate filter (0.2 µm pore size). Bar, 2 µm.

Na$^+$ ions were added together with divalent ions (Mg^{2+} or Ca^{2+}) or when Na$^+$, K$^+$, Mg^{2+} and Ca^{2+} were present in the medium. The salinity range that supported growth on diluted MA or in MA supplemented with NaCl, as reported by Macián *et al.* (2005), was between 0.3 and 12 % (w/v) total salts. Thus, strain MED297^T is a slight halophile with complex ionic requirements. In contrast, *R. marinisedimentorum* CECT 7208^T and *S. impatiens* CECT 5721^T only require sodium ions and exhibit different salinity ranges (Table 1). Strain MED297^T is mesophilic, growing at 15–42 °C, but not at 4 or 45 °C on MA. Again, *R. marinisedimentorum* CECT 7208^T and *S. impatiens* CECT 5721^T have narrower ranges.

Strain MED297^T was found to be oxidase- and catalase-positive and unable to grow under anaerobic conditions through either glucose fermentation or nitrate respiration. The same results were obtained with *S. impatiens* CECT 5721^T. In the case of *R. marinisedimentorum* CECT 7208^T, anaerobic growth through glucose fermentation was obtained, in agreement with the original description (Romanenko *et al.*, 2004). Strain MED297^T was negative for arginine dihydrolase, ornithine decarboxylase and indole production from tryptophan. It was able to hydrolyse DNA, casein, starch and Tween 80, but not alginate, agar or lecithin. The strain was unable to grow on marine broth 2216 with 12 % (w/v) gelatin. The API ZYM (bioMérieux) gallery was used to determine the enzymic activities of the strain; the gallery was used according to the manufacturer's instructions, except that the cells were suspended in a 3.5 % sea salts solution prior to addition to the API ZYM strips, and the strips were incubated for 20 h at 26 °C. The range of enzymic activities for strain MED297^T was broader than that for *R. marinisedimentorum* CECT 7208^T, but similar to that for *S. impatiens* CECT 5721^T (Table 1).

The 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$_4$Cl, 0.33 mM K$_2$HPO$_4$.3H$_2$O and 0.1 mM FeSO$_4$.7H$_2$O on half-strength artificial seawater 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$_4$Cl. Compounds were added at 2 g l^{-1}. Positive-control plates were prepared with 5 g yeast extract l^{-1}, while negative-control medium consisted of BMA. Growth was monitored for 12 days. About half of the compounds tested enabled growth of strain MED297^T. The preferred sources were as follows, in decreasing order of preference: organic acids, sugars, alcohols and then amino acids. Compared to strain MED297^T, *S. impatiens* and *R. marinisedimentorum* used much narrower ranges of utilizable resources and showed different substrate preferences (Table 1).

The cellular fatty acid composition of strain MED297^T was determined by GLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany), as described previously (Kämpfer & Kroppenstedt, 1996). Analyses of the polar lipids and respiratory quinones of strain MED297^T were carried out by the Identification Service of the DSMZ and Dr B. J. Tindall (DSMZ). The results are presented in the species description and in Table 2. Although some differences can be found between the cellular fatty acid and polar lipid profiles of strain MED297^T and those of *R. marinisedimentorum* and *S. impatiens*, they all exhibited the same major quinone, Q-8, and the polar lipids phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. Strain MED297^T and *R. marinisedimentorum*, but not *S. impatiens*, contained phosphatidylinositol. In general, it seems that the chemotaxonomic data are in agreement with the phylogenetic results in the sense that strain MED297^T is more closely related to *R. marinisedimentorum* than to *S. impatiens*.

The low levels of 16S rRNA gene sequence similarity between strain MED297^T and any other established species warrants its consideration as a separate species. The organisms most closely related to strain MED297^T are *R. marinisedimentorum* DSM 15388^T and *S. impatiens* EL-105^T (95 and 94 % sequence similarity, respectively); all others were clearly more distant. As discussed above, all three organisms differ in terms of a significant number of phenotypic traits. Thus, two possibilities seem reasonable: one is that strain MED297^T represents a novel genus and species; the other, which is more conservative, is that MED297^T represents a novel species within one of these two genera (*Reinekea* or *Saccharospirillum*). Because the phylogenetic analyses revealed that strain MED297^T clustered consistently with *R. marinisedimentorum* DSM 15388^T in the first instance, and because these two organisms also shared some phenotypic traits, including chemotaxonomic ones, strain MED297^T should be classified within a novel species of the genus *Reinekea*, for which the name *Reinekea blandensis* sp. nov. is proposed.

Table 1. Phenotypic characteristics that differentiate strain MED297[T] from closely related species.

Strains: 1, MED297[T]; 2, *R. marinisedimentorum* CECT 7208[T]; 3, *S. impatiens* CECT 5721[T]. +, Positive; −, negative; w, weakly positive. Data were obtained in this study unless indicated otherwise. None of the strains produced indole from tryptophan or hydrolysed lecithin. All strains used D-glucose, D-fructose, D-galactose (weakly), maltose, cellobiose, sucrose, melibiose (weakly), glycerol and pyruvate as sole energy and carbon sources. None of the strains was able to use D-ribose, D-xylose, trehalose, L-rhamnose, amygdalin, D-glucuronate, D-galacturonate, *myo*-inositol, D-sorbitol, D-saccharate, D-glycerate, glycine, L-serine, L-threonine, *trans*-aconitate, L-citrulline, L-histidine, L-lysine, L-sarcosine, betaine or putrescine as sole energy and carbon sources. None of the strains showed trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase activities in the API ZYM system.

Characteristic	1	2	3
Cell morphology	Curved rods	Rods	Spirilla
Cell size (μm)	$0.3–0.7 \times 1.2–2.8$	$0.4–0.5 \times 1.5–1.7$	$0.5–1.0 \times 2.0–12.0$
Glucose fermentation in O/F medium	−	+	−
Growth in anaerobic jar	−	+*	−*
Nitrate reduction	−†	+	+
Salt range (%, w/v)	0.3–12	0.9–8‡	0.9–12‡
Temperature range (°C)	15–42	15–37‡	15–30‡
Hydrolysis of:			
Casein	+	−	+*
Gelatin	−†	−	+
Starch	+	−	w‡
Alginate	−	w*	−*
Tween 80	+	−*	−†‡
DNA	+	−*	−‡
Growth on:			
L-Arabinose	+	−	−*
D-Mannose	+	w‡	+
Lactose	+	−*	w‡
Salicin	w	−*	−‡
D-Gluconate	+	−	+*
N-Acetyl-D-glucosamine	+	−	+*
D-Mannitol	+	+	−*
Acetate	+	w*	+*
Citrate	+	−	w‡
Malate	w	−	+
Butyrate	w	−*	w*
2-Ketoglutarate	+	−*	+*
Succinate	+	−*	−‡
Fumarate	+	−*	−*
Malate	w	−	−‡
Lactate	+	−*	w*
DL-β-Hydroxybutyrate	+	−*	w*
Propionate	+	+*	w‡
L-Leucine	+	−*	w*
L-Glutamate	w	−*	w‡
L-Alanine	−	−*	w*
L-Arginine	+	−*	w*
L-Tyrosine	+	−*	w*
L-Ornithine	w	−*	w*
γ-Aminobutyric acid	−	−*	w*
L-Aspartate	−	−*	+*
DNA G+C content (mol%)	52.4	51.1[a]§	54.6[b]

*Traits not determined previously for reference strains.
†No growth in medium.
‡Differs from previously published result(s).
§Data taken from: *a*, Romanenko *et al.* (2004); *b*, Labrenz *et al.* (2003).

Table 2. Fatty acid compositions (%) of strain MED297^T and *R. marinisedimentorum* KMM 3655^T

Cells were grown on MA at 35 °C for 3 days. –, Not detected. Data for *R. marinisedimentorum* KMM 3655^T were taken from Romanenko *et al.* (2004).

Fatty acid	MED297^T	*R. marinisedimentorum* KMM 3655^T
14:0	<1	1.99
15:0	2.7	4.11
16:0	13.97	31.61
17:0	9.07	5.94
18:0	<1	<1
15:1ω6*c*	<1	–
16:1ω7*c*	21.48*	26.72
17:1ω6*c*	5.40	1.89
17:1ω8*c*	5.39	1.29
18:1ω7*c*	14.59	19.04
12:0 ALDE	<1	<1
14:0 iso	<1	–
15:0 iso	<1	–
16:0 iso	17.25	–
17:0 iso	<1	–
17:0 10-methyl	–	<1
18:0 iso	4.54	–
18:1 iso	1.28	–
14:0 3-OH	1.21	2.35
15:0 3-OH	<1	–
16:0 *N* alcohol	–	1.21
16:1ω7*c* alcohol	–	1.09

*Identified as 16:1ω7*c*/15:0 iso 2-OH (summed feature 3).

Description of *Reinekea blandensis* sp. nov.

Reinekea blandensis (blan.den′sis. L. fem. adj. *blandensis* pertaining to Blande or Blanda, the name the Romans used for the city of Blanes, which has given its name to the Bay of Blanes, where the type strain was isolated).

Gram-negative, strictly aerobic, chemo-organotrophic bacterium. Oxidase- and catalase-positive. Cells are straight or slightly bent motile rods, 0.3–0.7 µm in diameter and 1.2–2.8 µm long. Gas vesicles are not observed. Poly-β-hydroxybutyrate granules are produced. Carbohydrates are not fermented. Nitrate is not reduced to nitrite or N$_2$. At least 0.3 % (w/v) marine salts is required; up to 12 % (w/v) salts is tolerated. Positive for growth at 15–42 °C. No growth detected at 4 or 45 °C. Casein, starch, Tween 80 and DNA are hydrolysed. Does not hydrolyse gelatin (no growth in medium), alginate, agar or lecithin. Negative for arginine dihydrolase, ornithine decarboxylase and indole production from tryptophan. Utilizes the following compounds as carbon and energy sources: L-arabinose, D-glucose, D-fructose, D-mannose, maltose, cellobiose, sucrose, lactose, *N*-acetyl-D-glucosamine, glycerol, D-mannitol, D-gluconate, propionate, pyruvate, acetate, citrate, 2-ketoglutarate,

succinate, fumarate, lactate, DL-β-hydroxybutyrate, L-leucine, L-tyrosine and L-arginine. Weakly positive results are obtained on D-galactose, melibiose, salicin, butyrate, malate, L-glutamate and L-ornithine. Growth is negative on D-ribose, D-xylose, trehalose, L-rhamnose, amygdalin, D-glucuronate, D-galacturonate, *myo*-inositol, D-sorbitol, D-saccharate, D-glycerate, glycine, L-serine, L-threonine, L-alanine, *trans*-aconitate, γ-aminobutyric acid, L-citrulline, L-histidine, L-aspartate, L-lysine, L-sarcosine, betaine and putrescine. Major cellular fatty acids are, in decreasing order of abundance, 16:1ω7*c*/15:0 iso 2-OH, 16:0 iso, 18:1ω7*c* and 16:0. The whole pattern and relative abundances are given in Table 2. Polar lipids consist of phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, three unidentified phospholipids, an unknown aminolipid and a lipid. Main respiratory quinone is Q-8 (92 %); Q-9 is present in minor amounts. The DNA G+C content of the type strain is 52.4 mol%.

The type strain, MED297^T (=CECT 7120^T =CCUG 52066^T), was isolated from surface water from the Mediterranean Sea.

Acknowledgements

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