Thalassobacter stenotrophicus gen. nov., sp. nov., a novel marine α -proteobacterium isolated from Mediterranean sea water

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A Gram-negative, slightly halophilic, strictly aerobic, chemo-organotrophic bacterium was isolated from Mediterranean sea water near Valencia (Spain). 16S rRNA gene sequence comparisons showed that the isolate represented a separate branch within the α -3 subclass of the *Proteobacteria*, now included within the order '*Rhodobacterales*'. *Jannaschia helgolandensis* was the closest relative, but their low sequence similarity and other features indicated that they were not related at the genus level. Isolate 5SM22^T produced bacteriochlorophyll *a* and grew on solid media as regular salmon-pink colonies. Cells are motile rods, with polar flagella. The DNA G + C content is 59.1 mol%. Morphological, physiological and genotypic differences from related, thus far known genera support the description of *Thalassobacter stenotrophicus* gen. nov., sp. nov. with strain 5SM22^T (= CECT 5294^T = DSM 16310^T) as the type strain.

The number of taxa within the α -*Proteobacteria* has increased considerably in recent years. Originally, Woese *et al.* (1984) described the group based on five representatives: two photosynthetic species, *Rhodopseudomonas* (now *Rhodobacter*) *sphaeroides* and *Rhodopseudomonas capsulata* (now *Rhodobacter capsulatus*), *Paracoccus denitrificans* and two manganese-oxidizing isolates. Currently, this group includes more than 100 genera, with different lifestyles and metabolic abilities. According to their 16S rRNA gene phylogeny, they have been organized into groups named with numbers, from α -1 to α -4. One of these groups, the α -3-*Proteobacteria*, also called the 'marine alpha group' (González & Moran, 1997) or *Roseobacter* group, includes mainly bacteria isolated from marine environments or hypersaline lakes with diverse metabolisms (phototrophy, aerobic sulfite oxidation, organic sulfur

compound degradation and lignin degradation), thus indicating that these bacteria play an important role in carbon, sulfur and nitrogen cycling (González *et al.*, 1999, 2000). In the last release (Garrity *et al.*, 2003) of the taxonomic outline of the prokaryotes for *Bergey's Manual of Systematic Bacteriology*, the whole group was classified within the order '*Rhodobacterales*'.

Throughout the α -subclass, photosynthetic and non-photosynthetic phenotypes are completely intermixed. Obligate aerobic, bacteriochlorophyll *a* (Bchl *a*)-containing bacteria within the *Rhodobacteraceae* comprise *Roseivivax* (Suzuki *et al.*, 1999b), *Roseobacter* (Shiba, 1991), *Roseovarius* (Labrenz *et al.*, 1999), *Rubrimonas* (Suzuki *et al.*, 1999a) and *Staleyia* (Labrenz *et al.*, 2000), as well as many undescribed isolates (Allgaier *et al.*, 2003).

A slightly halophilic, pigmented, strictly aerobic bacterium was isolated from Mediterranean sea water and phenotypically characterized in a previous study (Ortigosa *et al.*, 1994). Strain 5SM22^T (= CECT 5294^T) clustered in the unidentified phenon 45. The present publication describes this isolate, which can produce Bchl *a* under aerobic conditions and represents a new genus and species, *Thalassobacter stenotrophicus* gen. nov., sp. nov.

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Abbreviations: Bchl *a*, bacteriochlorophyll *a*; PHB, polyhydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Thalassobacter stenotrophicus* CECT 5294^T is AJ631302.

Scanning electron micrographs of cell division and the results of cellular fatty acid analysis are available as supplementary data in IJSEM Online.

Cultures were maintained on marine agar (MA; Difco) slants at room temperature and as suspensions in marine broth 2216 (MB; Difco) plus 10 % glycerol at -80°C . They were routinely grown at $24\text{--}26^{\circ}\text{C}$ on MA or MB. Most methods for phenotypic characterization, including cellular fatty acid analysis, were performed as previously described (Macián *et al.*, 2001). Bacteria grown on MA were used for scanning electron microscopy. A cell suspension was prepared with half-strength artificial sea water (ASW) (400 mM NaCl, 100 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mM KCl and 20 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$; Baumann & Baumann, 1981) and adsorbed onto Isopore membrane filters. Filters were dehydrated with a graded series of ethanol (50, 80 and 100 %), critical-point dried with CO_2 (Autosamdri-814) and sputter-coated with a gold-palladium film to approximately 10 nm thickness (Sputter coater; Bio-Rad). 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 Emip 3.0.

Cells were ovoid to irregular rods and motile in young cultures by one polar flagellum that was not easily seen by optical microscopy. Division by central constriction and also by budding could be observed on scanning electron micrographs (see Supplementary Figure in IJSEM Online). Rosette formation was not observed. Bright granules were never seen on wet mounts of cells of different ages and culture conditions, suggesting that strain 5SM22^T does not accumulate polyhydroxybutyrate (PHB).

The organism was unable to ferment sugars under anaerobic conditions as determined on anaerobic Hugh & Leifson O/F medium with half-strength ASW. It did not reduce nitrate to nitrite in nitrate broth and was also unable to grow in the denitrification medium of Baumann (Baumann & Baumann, 1981). Thus, it is a strict aerobe.

Growth at different temperatures was tested in both liquid (MB) and solid (MA) media for 7 days at 4, 13, 20, 28, 37 and 40°C . Strain 5SM22^T was unable to grow at 4 or 40°C , whereas good growth and pigmentation were observed between 13 and 37°C .

The ionic requirements of strain 5SM22^T were determined in salt-tolerance agar (STA) containing 1 % (w/v) tryptone, 0.3 % (w/v) yeast extract and 1.5 % (w/v) agar with the following combinations of salts: (i) STA with 2 % (w/v) NaCl; (ii) STA with 2 % (w/v) NaCl, 0.9 % (w/v) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 % (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.06 % (w/v) KCl (all concentrations are equal to those found in MB for these four salts); and (iii) STA with 2 % (w/v) NaCl, 0.9 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 % (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.06 % (w/v) KCl. Strain 5SM22^T was unable to grow after incubation for 7 days at 25°C in STA with NaCl, indicating that the Na^+ ion alone was not sufficient to meet the saline requirements of this organism. Moreover, the addition of the other three major cations of sea water as chloride salts or the replacement of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ by $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was

not sufficient to enable growth under the same incubation conditions. For comparative purposes, the type strain of *Jannaschia helgolandensis*, DSM 14858^T, was included in the ionic requirements tests; this strain showed good growth in STA with four added salts (media ii and iii). Therefore, strain 5SM22^T has more complex ionic requirements than *J. helgolandensis* DSM 14858^T.

For comparison of growth on another common medium for marine organisms, strain 5SM22^T was inoculated on basal medium agar (BMA) plus 0.5 % (w/v) yeast extract. BMA contains 50 mM Tris/HCl, pH 7.5, 19 mM NH_4Cl , 0.33 mM $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.1 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 % (w/v) purified agar (Oxoid) in half-strength ASW (Baumann & Baumann, 1981). Good growth was obtained.

The range of salinities that support growth of strain 5SM22^T was tested in MA. In order to determine the minimal amount of salts required for growth, MA was diluted with distilled water to produce media containing 0.34, 0.68, 0.85, 1.02, 1.36, 1.7, 2.04, 2.55 and 3.06 % (w/v) total salts (dilution factors 0.1, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.75 and 0.9, respectively). The loss of nutrients and agar by dilution was compensated for by adding the appropriate amounts of peptone, yeast extract and agar. A progressive diminution of growth was observed from 3.06 to 1.02 % (w/v) compared with growth in MA. Pigmentation was also delayed. At 0.85 % (w/v) salts, weak growth could only be observed after incubation for 7 days and pigmentation did not occur. The highest salinity that supported growth was determined on MA plus NaCl up to 6, 7, 8 and 9 % (w/v) total salinity [plus 2.6, 3.6, 4.6 and 5.6 % (w/v) NaCl, respectively]. Growth after incubation for 7 days occurred in media containing 6 and 7 % (w/v) total salinity, but pigmentation was absent with 7 % (w/v) total salinity. No growth was observed with 8 and 9 % (w/v) salts.

Nutritional screening was carried out on BMA supplemented with 0.1 g yeast extract l^{-1} since the organism was originally reported to be unable to grow on minimal medium (Ortigosa *et al.*, 1994). Fifty-six compounds, including carbohydrates, amino acids, organic acids and amines, were tested. Carbohydrates were added at 2 g l^{-1} , while the rest of compounds were added at 1 g l^{-1} . Positive-control plates were prepared with 5 g yeast extract l^{-1} , while negative-control plates consisted of BMA plus 0.1 g yeast extract l^{-1} . Growth was scored as negative when growth was equal to or less than the negative-control plates. The ability to grow using these substrates is given in Table 1 and in the species description.

Bchl *a* production was determined in acetone extracts using a Beckman DU-600 spectrophotometer as previously described (Takaichi *et al.*, 1991). Extracts obtained from cells grown in the dark on BMA plates containing one of the carbon sources that support growth were analysed for the presence of Bchl *a*. The absorbance spectrum of 5SM22^T revealed a large peak at 772 nm and smaller peaks at 578 and 750 nm, characteristic of the presence of Bchl *a*.

Table 1. Phenotypic characteristics that differentiate *Thalassobacter stenotrophicus* gen. nov., sp. nov. from related genera

Genera: 1, *Thalassobacter* gen. nov.; 2, *Jannaschia*; 3, *Ruegeria*; 4, *Octadecabacter*; 5, *Sulfitobacter*; 6, *Staleyia*; 7, *Roseobacter*; 8, *Leisingeria*; 9, *Roseovarius*. Abbreviations: +, only positive results reported; –, only negative results reported; w, only weakly positive results reported; v, varies between species and/or strains; ND, not determined; P, pink to red or orange; N, none (colonies may appear cream, whitish, beige or brown coloured). Data are from this study, González *et al.* (2003), Gosink *et al.* (1997), Ivanova *et al.* (2004), Labrenz *et al.* (1999, 2000), Lafay *et al.* (1995), Pukall *et al.* (1999), Ruiz-Ponte *et al.* (1998), Rüger & Höfle (1992), Schaefer *et al.* (2002), Shiba (1991), Sorokin (1995), Uchino *et al.* (1998) and Wagner-Döbler *et al.* (2003).

Characteristic	1	2	3	4	5	6	7	8	9
Number of species	1	1	3	2	5	1	3	1	2
Oxidase	+	w	+	–	+	+	+	+	v
Pigmentation	P	N	N	N	N	P	N, P	N	P
Motility	+	–	v	–	v	+	+	+	+
Cell shape	Ovoid rods	Irregular rods	Rods, ovoid rods	Rods	Rods, short fusoid rods	Short fusoid rods	Rods, ovoid rods	Rods, ovoid rods	Rods
PHB	–	+	–	ND	+	+	ND	ND	+
Bud formation	+	ND	–	–	+	+	–	ND	+
Rosettes	–	–	ND	ND	+	+	ND	–	ND
Bchl <i>a</i>	+	–	–	–	–	v	v	ND	+
Sea salts requirement	+*	+*	+	ND	–	–	–	ND	ND
Salt range (%)	0.85–7	1–7	0.6–12	1.8–7	0.2–15	1–15	0.6–12	1.0–6.5	1.0–13.0
Growth at 4 °C	–	–	–	+	v	+	+	–	+
Growth at 37 °C	+	–	+	–	v	–	+	–	+
Utilization of:									
D-Glucose	–	+	v	w	v	w	+	–	v
D-Fructose	–	+	+	–	+	w	+	–	–
D-Galactose	–	+	v	ND	+	–	+	–	ND
D-Trehalose	–	–	v	ND	+	w	v	ND	ND
D-Mannose	–	+	v	ND	v	–	+	ND	ND
Maltose	–	–	v	ND	+	–	v	ND	ND
Cellobiose	–	+	v	ND	w	–	v	ND	v
Sucrose	–	–	v	–	+	w	v	ND	ND
D-Gluconate	–	+	v	ND	v	–	v	ND	–
Glycerol	–	+	v	ND	v	+	+	–	–
D-Mannitol	–	+	v	ND	v	–	+	ND	–
Pyruvate	+	–	+	v	+	+	+	–	+
Citrate	–	+	v	v	v	–	v	–	–
Malate	–	+	+	ND	+	+	+	–	+
Acetate	+	–	+	–	+	+	v	–	+
L-Glutamate	+	–	v	+	+	+	+	–	v
Presence of 19:0 cyclo	–	+	ND†	–	–	–	–/ND	ND	–
G + C content (mol%)	59.1	63.0–63.1	55–65	56–57	58–63.5	55.0–56.3	56.3–59.6	60.5	62.3–66.0

**J. helgolandensis* requires Na⁺, K⁺, Ca²⁺ and Mg²⁺ whereas *T. stenotrophicus* needs additional ions for growth.

†A complete list of cellular fatty acids is not available.

Cellular fatty acid analysis was performed by GLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) using a method described previously (Kämpfer & Kroppenstedt, 1996) and gave the results shown in a Supplementary Table in IJSEM Online.

The G + C content of the genomic DNA was 59.1 mol% as determined from the midpoint value of the thermal denaturation profile (Marmur & Doty, 1962) and procedures previously described by Macián *et al.* (2001).

To investigate the genealogy of our isolate, comparative 16S rRNA gene sequence analysis was performed. Isolation of genomic DNA, amplification of almost full-length 16S rRNA gene fragments and sequencing of the rRNA gene using a LICOR automated sequencer (MWG Biotech) were performed as previously reported (Macián *et al.*, 2001). Sequences were added to the 16S rRNA gene sequence databases of the Technical University Munich using the program package ARB (Ludwig *et al.*, 2004). Automated sequence alignments were inspected by eye and corrected

manually using the sequence editor ARB_EDIT. Phylogenetic analyses using alternative treeing methods (maximum parsimony, maximum likelihood and distance matrix) and data subsets were performed using the appropriate ARB tools to test the robustness of local topologies (Ludwig *et al.*, 1998). As is regularly the case in such tree comparisons, variations in topology could be detected, but none of them occurred among the closest relatives of our isolate. Fig. 1 shows the tree derived by distance-matrix analysis, using the Jukes–Cantor correction.

Comparative analysis of the sequence obtained in this study (GenBank/EMBL/DDBJ accession no. AJ631302) confirmed the affiliation of strain 5SM22^T to the α -subclass of the *Proteobacteria* and more precisely to a branch within the α -3-*Proteobacteria*. The closest 16S rRNA gene sequences from established species all corresponded to strains of the recently described *J. helgolandensis* (Wagner-Döbler *et al.*, 2003) (AJ438157, AJ534224 and AJ534225), bearing 95.7 % sequence similarity to strain 5SM22^T (Fig. 1). All other sequences from species belonging to the α -subclass of *Proteobacteria* showed levels of similarity below 95 %, the only exception being *Ruegeria gelatinovorans* (95.3 %). However, in all tree topologies examined, this organism clustered separately from our isolate, excluding the possibility of considering both organisms as members of the same genus.

The question of whether our isolate represented a novel

species of the genus *Jannaschia* or a separate genus was addressed by taking into consideration the distinct phenotypic and genetic properties of the two and the degree of difference found compared with neighbouring genera (Table 1). Phenotypic differences supporting the consideration of strain 5SM22^T as a member of a new genus included the ability to synthesize Bchl *a* and its characteristic pigmentation, motility and flagella arrangement, mode of division, a more complex saline requirement and the inability to produce PHB. In addition, there were important differences in the cellular fatty acid composition: the second most abundant fatty acid in *Jannaschia*, 19:0 cyclo (22–25 %), was absent in strain 5SM22^T. In addition, fatty acids 18:1 ω 9c and 20:1 ω 7c were minor components in strain 5SM22^T but absent in *Jannaschia*. The phylogenetic distance determined for the 16S rRNA gene sequences of strain 5SM22^T and *J. helgolandensis* was 4.3 %, which was higher than the distances calculated between some recently described neighbouring genera, such as *Silicibacter* and *Ruegeria* (as low as 1.7 %), *Leisingera* and *Roseobacter* (2.4 %), *Roseobacter* and *Sulfitobacter* (2.4 %), *Leisingera* and *Ruegeria* (3.2 %), *Roseibium* and *Stappia* (3.3 %) and *Loktanella* and *Jannaschia* (4.2 %). Table 1 indicates traits useful for distinguishing our isolate phenotypically and genetically from *Jannaschia* and other related organisms. In all cases, the number and taxonomic weight of the differences that were observed led us to propose isolate 5SM22^T as representing a new genus and species, *Thalassobacter stenotrophicus* gen. nov., sp. nov.

Fig. 1. 16S rRNA gene distance matrix-based tree (Jukes–Cantor correction) showing the phylogenetic relationship of *T. stenotrophicus* gen. nov., sp. nov. and other α -*Proteobacteria*. Accession numbers are shown in parentheses. The outgroup (more than 200 bacterial sequences) has been deleted to simplify the figure. Bar, 5 % sequence divergence.

Description of *Thalassobacter* gen. nov.

Thalassobacter (Tha.las'so.bac.ter. Gr. n. *Thalassa* the sea; N.L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod or staff; N.L. masc. n. *Thalassobacter* a bacterium of the sea).

Gram-negative, strictly aerobic, chemo-organotrophic, slightly halophilic bacteria. Oxidase and catalase positive. Cells are ovoid to irregular rods, approximately 0.5–2.0 µm in length by 0.5 µm in width, motile by polar flagella, and can be observed individually or in pairs but not as rosettes. They show budding and binary division. No growth can be obtained without sea water or the addition of combined marine salts to the medium. Mesophilic. No anaerobic growth with exposure to light can be observed. Gas vesicles or PHB accumulation are not observed. Bchl *a* is produced. They do not hydrolyse casein, gelatin, starch, lecithin, alginate, DNA, Tween 80 or agar. Enzymic activities of arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not detected. Cellular fatty acids include 18:1ω7c, 11-methyl 18:1ω7c, 18:1ω9c and 20:1ω7c. The genus is affiliated to the α-Proteobacteria, order 'Rhodobacterales', and so far contains a single species, *Thalassobacter stenotrophicus*, which is the type species.

Description of *Thalassobacter stenotrophicus* sp. nov.

Thalassobacter stenotrophicus (ste.no.tro'phi.cus. Gr. adj. *stenos* narrow; Gr. adj. *trophikos* nursing, tending or feeding; N.L. masc. adj. *stenotrophicus* feeding on a narrow range of compounds).

In addition to the characteristics that define the genus, the type species has the following characteristics. Growth on MA leads to regular, salmon-pink colonies that do not adhere to the agar. Pigment formation starts at the edges of the colonies or agar growing masses. Does not swarm or luminesce. Requires at least 0.85 % (w/v) marine salts and tolerates up to 7 % (w/v) salts, failing to grow at 8 %. Temperature range for growth is 13–37 °C and is optimum at 22–26 °C. No growth is detected at 4 or 40 °C. The DNA G+C content of the type strain is 59.1 mol%. Does not reduce nitrate to nitrite. Also negative for H₂S production from thiosulfate, indole production from tryptophan and sulfite oxidation (González *et al.*, 1999). No growth is observed on TCBS agar. Utilizes the following compounds as carbon and energy source: pyruvate, acetate, 2-ketoglutarate, succinate, DL-β-hydroxybutyrate, γ-aminobutyric acid, L-glutamate, L-ornithine, citrulline, sarcosine and putrescine, provided that the medium is supplemented with a small amount of yeast extract (an indication that undetermined growth factors are required). Under the same conditions, the following substrates are not utilized: D-ribose, D-glucose, D-fructose, L-arabinose, D-xylose, D-galactose, D-trehalose, D-mannose, L-rhamnose, maltose, cellobiose, sucrose, lactose, D-melibiose, D-raffinose, salicin, amygdalin, D-gluconate, D-glucuronate, D-galacturonate, N-acetylglucosamine, glucosamine, glycerol, D-mannitol,

D-sorbitol, meso-inositol, glycerate, fumarate, propionate, citrate, cis-aconitate, malate, lactate, p-hydroxybenzoate, D-saccharic acid, glycine, L-leucine, L-serine, L-arginine, L-alanine, L-threonine, L-aspartate, L-lysine, L-tyrosine and L-histidine.

The type strain, 5SM22^T (=CECT 5294^T=DSM 16310^T), was isolated from Mediterranean sea water.

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