Thalassococcus halodurans gen. nov., sp. nov., a novel halotolerant member of the *Roseobacter* clade isolated from the marine sponge *Halichondria panicea* at Friday Harbor, USA

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A Gram-negative, non-pigmented, ovoid-shaped, strictly aerobic, catalase- and oxidase-positive and highly halotolerant bacterial strain that was devoid of swimming and gliding motility, designated UST050418-052^T, was isolated from the surface of the marine sponge *Halichondria panicea* at Friday Harbor, WA, USA. Strain UST050418-052^T required NaCl for growth and could tolerate salt concentrations of up to 18%. The primary respiratory quinone was ubiquinone-10 and the DNA G + C content was 57.8 mol%. The predominant fatty acids were the saturated fatty acids 16:0 and 18:0 and the monounsaturated fatty acids 18:1 ω 7c and 18:1 ω 9c, altogether representing 82.9% of the total. Phylogenetic analysis based on the 16S rRNA gene sequence placed UST050418-052^T in a distinct lineage within the *Roseobacter* clade in the family *Rhodobacteraceae*, with 95.0–95.8% sequence similarity to members of the nearest genus *Thalassobius*. The DNA–DNA relatedness between UST050418-052^T and *Thalassobius gelatinovor* IAM 12617^T was 9%. Strain UST050418-052^T could be differentiated from closely related members of the *Roseobacter* clade by a number of chemotaxonomic and phenotypic characteristics such as its distinct fatty acid profile, ability to reduce nitrate to nitrite and inability to utilize citrate, succinate, L-arginine and pyruvate. Based on the phylogenetic, chemotaxonomic and phenotypic evidence presented in this study, we suggest that strain UST050418-052^T represents a novel genus in the family *Rhodobacteraceae*. The name *Thalassococcus halodurans* gen. nov., sp. nov., is thus proposed. The type strain of *Thalassococcus halodurans* is UST050418-052^T (=JCM 13833^T =NRRL B-41465^T).

The *Roseobacter* clade falls within the α -3 subgroup of the *Proteobacteria* and is one of the nine dominant marine clades that comprise marine microbial communities (Giovannoni & Rappé, 2000). Members of this clade represent one of the most readily cultivated groups of marine lineages and are isolated from diverse marine environments ranging from coastal to open waters, sea floor to sea ice, in the form of free-living or particle-attached cells or in association with marine phytoplankton, invertebrates or vertebrates (see review by Buchan *et al.*, 2005 and references therein). The first genus within this clade, *Roseobacter*, was established in 1991, in which two

species, *Roseobacter litoralis* and *Roseobacter denitrificans*, were described (Shiba, 1991). Since then, the number of species and genera described has been increasing steadily. By 2004, this clade contained 35 species with validly published names from 16 genera: *Antarctobacter*, *Jannaschia*, *Ketogulonicigenium*, *Leisingera*, *Loktanelia*, *Oceanibulbus*, *Oceanicola*, *Octadecabacter*, *Roseobacter*, *Roseovarius*, *Ruegeria*, *Sagittula*, *Salipiger*, *Silicibacter*, *Staleyia* and *Sulfitobacter* (Schaefer *et al.*, 2002; Allgaier *et al.*, 2003; Cho & Giovannoni, 2004; Martínez-Cánovas *et al.*, 2004; Wagner-Döbler *et al.*, 2004; Van Trappen *et al.*, 2004). At the time of writing, about 45 species have been described in 24 genera (Yi & Chun, 2006). This paper deals with the description of another novel bacterial strain in the *Roseobacter* clade, strain UST050418-052^T.

Strain UST050418-052^T was isolated from the surface of a colony of the marine sponge *Halichondria panicea* collected

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain UST050418-052^T is DQ397336.

Scanning electron micrographs of cells of strain UST050418-052^T are available as supplementary material with the online version of this paper.

from Friday Harbor, San Juan Island, WA, USA, in April 2005. A freshly collected sponge colony was rinsed with autoclaved 0.22 µm-filtered seawater (AFSW) to remove loosely attached bacteria and its surface was then swabbed with a sterile cotton bud, which was then placed in a culture tube containing 1 ml AFSW for subsequent bacterial isolation and purification procedures. By using the standard dilution plating technique on a marine agar medium containing 3 g yeast extract (Oxoid), 5 g peptone (Oxoid) and 12 g bacteriological agar (Oxoid) in 1 l AFSW at 32‰ salinity and repeated purification and incubation at 28 °C for 48 h, pure colonies of the strain were obtained. Unless otherwise specified, all characteristics described hereafter were based on cultures grown on marine agar under the same conditions. Pure colonies were observed under a light microscope (Leica MZ6; × 40 magnification) as milky, raised and circular (1.1–1.4 mm in diameter) with an entire edge and a smooth surface. Gram staining was determined using light microscopy according to Smibert & Krieg (1994) and cell morphology was examined using scanning electron microscopy (6700F; JEOL) according to Neu *et al.* (2001). Swimming and gliding motilities were observed under a phase-contrast light microscope (Olympus BX51; × 100 magnification) after growing the strain on one-quarter-strength marine broth 2216 (Oxoid) solidified with 0.4 or 1.2% agar, respectively (Bowman, 2000). Strain UST050418-052^T appeared as Gram-negative, non-motile and ovoid-shaped cells (see Supplementary Fig. S1 available in IJSEM Online).

The nearly complete 16S rRNA gene sequence of strain UST050418-052^T (1383 bp) was obtained bidirectionally with three replicates as described by Lau *et al.* (2004). Comparative analysis of the 16S rRNA gene sequence with sequences deposited in GenBank using BLAST indicated that the strain fell within the *Roseobacter* clade in the α-subgroup of the *Proteobacteria* and shared the highest sequence similarities, 99.6–99.8%, with five uncharacterized *Roseobacter* strains (strains JL-129, JL-131, JL-132, JL-135 and JL-137). The most closely related characterized relatives of UST050418-052^T are members of the genera *Thalassobius* (Arahal *et al.*, 2005; Yi & Chun, 2006), *Phaeobacter* (Martens *et al.*, 2006), *Marinovum* (Martens *et al.*, 2006) and *Leisingera* (Schaefer *et al.*, 2002), with 95.0–95.8% sequence similarity. The 16S rRNA gene sequence was aligned automatically and then manually with a database of >30 000 already-aligned 16S rRNA gene sequences using the ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees showing the relatedness of strain UST050418-052^T and other close members in the *Roseobacter* clade were then constructed using three tree-drawing methods: neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971). The neighbour-joining tree showed that strain UST050418-052^T formed a distinct cluster with the five uncharacterized *Roseobacter* strains, and this cluster was distantly related to another cluster comprising the members of *Thalassobius* (Fig. 1). This close phylogenetic relationship was also revealed in the

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships among strain UST050418-052^T and related species based on 16S rRNA gene sequence comparison. Strains belonging to the genus *Paracoccus* were chosen as the outgroup. Filled circles indicate nodes also found in the maximum-parsimony and maximum-likelihood trees. Bootstrap values over 50% (from 500 replicates) are shown at nodes. GenBank accession numbers are shown in parentheses. Bar, 1 substitution per 100 nucleotide positions.

Table 1. Cellular fatty acid composition of strain UST050418-052^T and members of closely related genera

Strains: 1, UST050418-052^T; 2, *Thalassobius aestuarii* JC2049^T (data from Yi & Chun, 2006); 3, *Thalassobius gelatinovorius* IAM 12617^T (Arahal *et al.*, 2005); 4, *Thalassobius mediterraneus* XSM19^T (Arahal *et al.*, 2005); 5, *Phaeobacter inhibens* T5^T (Martens *et al.*, 2006); 6, *Phaeobacter gallaeciensis* BS107^T (Martens *et al.*, 2006); 7, *Leisingera methylohalidivorans* MB2^T (Martens *et al.*, 2006); 8, *Marinovum algicola* ATCC 51440^T (Martens *et al.*, 2006). Values given are mean percentages of total fatty acids. —, Not detected. Fatty acids representing <1% in all rows were excluded.

Fatty acid	1	2	3	4	5	6	7	8
Saturated								
10:0	—	—	3.5	—	—	—	—	—
12:0	—	—	3.1	—	—	—	—	—
16:0	13.9	6.8	2.9	3.0	—	—	—	—
17:0	0.7	1.4	—	—	3.8	3.8	5.1	—
18:0	10.9	3.0	1.9	1.1	—	—	—	—
10:0 3-OH	—	1.3	1.5	1.9	3.1	2.6	1.0	2.7
12:0 3-OH	—	—	5.8	—	1.7	1.9	1.8	1.2
16:0 2-OH	—	3.8	—	—	1.6	1.3	2.3	1.6
19:0 cyclo	—	—	2.3	—	3.1	3.9	7.0	—
Unsaturated								
12:1 3-OH	3.2	—	—	4.0	—	—	—	—
18:1 ω 9 c	21.0	—	—	—	0.8	0.9	2.5	0.8
18:1 ω 7 c	37.1	68.0	68.8	84.6	2.2	2.1	2.3	—
18:1 ω 7 c	3.1	12.0	7.5	—	0.8	0.9	2.5	0.8
11-methyl								
20:2 ω 6,9 c	1.3	—	—	—	73.8	75.5	70.3	86.7
Summed feature 3*	1.2	—	—	—	7.5	6.6	6.9	3.2
Summed feature 5*	5.9	—	—	—	—	—	—	—

*Summed feature 3 comprises iso-15:0 2-OH and/or 16:1 ω 7 c . Summed feature 5 comprises 18:2 ω 6,9 c and/or anteiso-18:0.

maximum-parsimony and maximum-likelihood trees (data not shown). Strain UST050418-052^T also shared the same lineage with the members of *Phaeobacter*, *Marinovum* and *Leisingera*. DNA–DNA hybridizations were performed by the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, University Gent, Gent, Belgium) as described by Ezaki *et al.* (1989) to determine the relatedness of UST050418-052^T with its close relatives. The strain showed DNA relatedness with *Thalassobius gelatinovorius* IAM 12617^T and *Phaeobacter inhibens* T5^T of 9 and 7%, respectively, which clearly indicated that UST050418-052^T does not belong to the genera *Thalassobius* or *Phaeobacter*. These results support the conclusion that UST050418-052^T represents a novel genus within the *Roseobacter* clade.

Chemotaxonomic characteristics including the DNA G+C content, respiratory quinone and cellular fatty acid profile of UST050418-052^T were compared with those of members of the closely related genera *Thalassobius*, *Phaeobacter*, *Marinovum* and *Leisingera*. The DNA G+C content of

UST050418-052^T was determined by using an HPLC method as described in Mesbah *et al.* (1989) as 57.8 ± 0.1 mol% ($n=3$). This value is within the range of the G+C contents (57–61 mol%) observed among the members of closely related genera (Table 2). Extraction of respiratory quinones followed Minnikin *et al.* (1984) and the presence of ubiquinone Q-10, the primary isoprenoid quinone found in *Thalassobius*, *Phaeobacter*, *Marinovum* and *Leisingera*, was verified using an HPLC method according to Collins (1994). Ubiquinones extracted from *Agrobacterium tumefaciens* ATCC 23308^T as described above served as a reference for Q-10. The cellular fatty acid profile of the strain was determined using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's protocol. Strain UST050418-052^T had a relatively simple cellular fatty acid profile which was dominated by the monounsaturated fatty acids 18:1 ω 7 c (37.1%) and 18:1 ω 9 c (21.0%), followed by the saturated fatty acids 16:0 (13.9%) and 18:0 (10.9%), altogether representing 82.9% of the total fatty acids (Table 1). The most dominant fatty acid in UST050418-052^T, 18:1 ω 7 c , was also found in members of *Thalassobius*, *Phaeobacter* and *Leisingera*, but the relative proportions were drastically different; the other dominant fatty acids, 18:1 ω 9 c , 16:0 and 18:0, were found in very small proportions or even not detected in its close relatives (Table 1). On the other hand, the polyunsaturated fatty acid 20:2 ω 6,9 c was found in large proportions in *Phaeobacter*, *Leisingera* and *Marinovum* (70.3–86.7%) but not in UST050418-052^T. Results from fatty acid analysis therefore differentiated UST050418-052^T from closely related genera.

Phenotypic characteristics including growth conditions, susceptibility to antibiotics, hydrolysis of various substrates, reduction of nitrate, production of enzymes, H₂S, indole and acetoin and utilization of and acid production from carbon sources were studied. The requirement for oxygen for growth was examined using the Oxoid Anaerobic System. Growth at different temperatures (4–52 °C at intervals of 8 °C) and pH (pH 5–10 at intervals of 1 pH unit) was monitored on marine agar for up to 10 days of incubation, while growth at different salt concentrations [0–18% (w/v) NaCl at intervals of 2%] was tested on a 1.2% agar medium containing 5 g peptone, 5 g MgCl₂, 2 g MgSO₄, 1 g KCl, 0.5 g CaCl₂ and different amounts of NaCl, with pH adjusted to 7.5 using KOH (Isnansetyo & Kamie, 2003). Susceptibility to streptomycin, benzylpenicillin, chloramphenicol, ampicillin, tetracycline and kanamycin (amount loaded onto paper discs ranged from 1.0 to 100.0 µg per disc) was studied using standard agar disc-diffusion assays according to Acar (1980). Hydrolysis of casein and cellulose was tested according to Norris *et al.* (1985) and Bowman (2000), respectively. Hydrolysis of Tweens 20, 40 and 80 and chitin was tested as described by Baumann & Baumann (1981). Hydrolysis of agar, DNA and starch and production of oxidase and catalase were determined according to Smibert & Krieg (1994). Other enzyme activities and the ability to reduce nitrate, produce

H₂S, indole and acetoin and utilize different carbon sources were assessed using commercial API 20E, API 20NE, API 50CH and API ZYM systems (bioMérieux) and MicroLog 3 (Biolog) according to the manufacturers' manuals. Cells for inoculating into the API 20E system were suspended in sterile seawater at 22‰ salinity before inoculation (MacDonell *et al.*, 1982). Growth on glycerol, D-glucose, sucrose, D-mannitol, D-galactose, starch, D-sorbitol, D-arabinose and D-melibiose as sole carbon sources was also examined on a 1.2% agar medium containing 0.2 g

NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract and 4% (w/v) carbon source per litre seawater at 35‰ salinity (Nedashkovskaya *et al.*, 2003). Detailed physiological and biochemical characteristics of UST050418-052^T are given in the species description.

UST050418-052^T differs from most of its close relatives by its ability to grow at salt concentrations higher than 9% and at 40 °C and to reduce nitrate to nitrite and its inability to utilize citrate, succinate, L-arginine and

Table 2. Phenotypic characteristics that differentiate UST050418-052^T from members of closely related genera

Strains: 1, UST050418-052^T; 2, *Thalassobius aestuarii* JC2049^T; 3, *Thalassobius gelatinovor* IAM 12617^T; 4, *Thalassobius mediterraneus* XSM19^T; 5, *P. inhibens* T5^T; 6, *P. gallaeciensis* BS107^T; 7, *L. methylohalidivorans* MB2^T; 8, *M. algicola* ATCC 51440^T. Data were taken from this study, Ruiz-Ponte *et al.* (1998), Uchino *et al.* (1998), Schaefer *et al.* (2002), Arahal *et al.* (2005), Martens *et al.* (2006) and Yi & Chun (2006). All strains are Gram-negative, strictly aerobic, require Na⁺ for growth and are positive for catalase and oxidase activities. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
DNA G + C content (mol%)	58	61	59	57	56	58	61	60
Cell shape	Ovoid	Ovoid	Rod	Coccoid/rod	Ovoid rod	Ovoid rod	Rod	Ovoid
Motility	–	–	+	–	+	+	+	+
Pigment	–	–	–	–	Brown	Brown	–	Pink
Growth at/in:								
4 °C	–	+	+	–	+	–	+	–
40 °C	+	–	+	–	–	–	–	–
1% NaCl	+	+	+	–	+	+	+	+
8% NaCl	+	–	ND	+	+	+	–	+
9% NaCl	+	–	ND	–	–	+	–	+
Nitrate reduction to nitrite	+	–	+	–	–	–	–	–
Nitrite reduction to nitrogen	–	+	–	–	–	–	–	–
Hydrolysis of:								
Gelatin	–	+	+	–	–	–	–	+
Tween 80	–	–	+	–	–	–	–	–
Production of:								
Valine arylamidase	+	–	+	–	ND	ND	ND	ND
Acid phosphatase	+	+	+	–	ND	ND	ND	ND
Naphthol-AS-BI-phosphohydrolase	+	+	–	–	ND	ND	ND	ND
α-Galactosidase	+	–	–	–	ND	ND	ND	ND
β-Galactosidase	+	–	+	–	ND	ND	ND	ND
Lysine decarboxylase	–	+	ND	–	ND	ND	ND	ND
Ornithine decarboxylase	–	+	ND	–	ND	ND	ND	ND
Utilization of:								
Glycerol	+	+	+	+	+	+	–	–
D-Ribose	–	+	+	+	+	ND	ND	–
D-Xylose	+	+	ND	–	+	+	ND	–
D-Glucose	+	+	+	+	+	+	–	+
D-Fructose	+	–	+	+	+	+	–	+
D-Mannitol	+	–	+	+	+	+	ND	–
D-Sorbitol	+	–	+	+	+	+	–	–
D-Cellobiose	+	–	ND	–	+	+	–	–
Sucrose	+	–	+	+	+	+	ND	–
Trehalose	+	–	–	ND	+	+	–	+
Citrate	–	+	+	+	+	–	–	+
Succinate	–	+	+	+	+	+	–	–
L-Arginine	–	+	+	+	+	+	ND	+
Pyruvate	–	ND	+	+	+	ND	–	+

pyruvate (Table 2). Apart from these characteristics, the strain can be further differentiated from its closest relative *Thalassobius aestuarii* JC2049^T by its ability to grow at 8 % NaCl, produce valine arylamidase, α - and β -galactosidases and to utilize D-fructose, D-mannitol, D-sorbitol, D-cellobiose, sucrose and trehalose as sole carbon sources and its inability to grow at 4 °C, to reduce nitrite to nitrogen, to hydrolyse gelatin, to produce lysine decarboxylase and ornithine decarboxylase and to utilize D-ribose. It also differs from other close relatives by a number of phenotypic characteristics as shown in Table 2. Based on the phylogenetic evidence and chemotaxonomic and phenotypic characteristics presented in this study, we suggest that strain UST050418-052^T represents a novel member in the *Roseobacter* clade and propose the name *Thalassococcus halodurans* gen. nov., sp. nov.

Description of *Thalassococcus* gen. nov.

Thalassococcus (Tha.las.so.coc'cus. Gr. n. *thalassa* the ocean; L. n. *coccus* from Gr. n. *kokkos* berry, coccus; N.L. masc. n. *Thalassococcus coccus* from the ocean).

Cells are Gram-negative, ovoid-shaped, non-pigmented, devoid of swimming and gliding motility, strictly aerobic and halophilic. Oxidase- and catalase-positive. The major respiratory quinone is ubiquinone Q-10. The major fatty acids are 16:0, 18:0, 18:1 ω 9c, 18:1 ω 7c (altogether representing 82.9 % of the total in the type strain of the type species) and summed feature 5 (18:2 ω 6,9c and/or anteiso-18:0). Phylogenetic analysis based on 16S rRNA gene sequences suggests that *Thalassococcus* is a member of the *Roseobacter* clade in the family *Rhodobacteraceae*. The genus currently contains one species, *Thalassococcus halodurans*, the type species.

Description of *Thalassococcus halodurans* sp. nov.

Thalassococcus halodurans (ha.lo.du'rans. Gr. n. *hals* salt, L. part. pres. *durans* withstanding, N.L. part. adj. *halodurans* withstanding salt).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.7–1.0 μ m in diameter and colonies are milky, 1.1–1.4 mm in diameter, raised and circular with a smooth surface and an entire edge when cultivated on marine agar at 28 °C for 48 h. Cells do not produce flexirubin-type or diffusible pigments. Growth occurs at pH 6–10 and 12–44 °C, with optimal growth at 28–36 °C; no growth below pH 5 or at 4 or 52 °C. Cells require a minimum of 2.0 % NaCl for growth and tolerate up to 18.0 % NaCl (optimum growth at 2.0–6.0 %). The DNA G+C content is 58.8 mol%. Cells are susceptible to 1.0 μ g benzylpenicillin, chloramphenicol and ampicillin, 5.0 μ g kanamycin and tetracycline and 10.0 μ g streptomycin. Does not hydrolyse starch, casein, chitin, cellulose, agar, gelatin or Tween 20, 40 or 80. Acetoin is produced but not indole or H₂S. Nitrate is reduced to nitrite but not

to nitrogen gas. Citrate is not utilized. Positive for activities of DNase, alkaline phosphatase, esterase (C₄), esterase lipase (C₈), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α - and β -galactosidases, α - and β -glucosidases and arginine dihydrolase. Negative for activities of urease, lipase (C₁₄), cystine arylamidase, trypsin, α -chymotrypsin, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase. Utilizes glycerol, D-glucose, sucrose, D-mannitol, D-galactose, starch, D-sorbitol, D-arabinose and D-melibiose as sole carbon sources on agar medium supplemented with 4 % (w/v) carbon source, glycerol, L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, inositol, D-mannitol, D-cellobiose, maltose, D-melibiose, sucrose, trehalose and potassium 2-ketogluconate in the API 50 CH system and D-sorbitol in the MicroLog 3 system. Utilization of other carbon sources included in the MicroLog 3, API 20 NE and 50 CH systems is not observed. Acid is produced from glycerol, L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, inositol, D-mannitol, D-sorbitol, D-melibiose, sucrose, D-fucose and potassium 2-ketogluconate in API 50 CH, but no acid production is observed in the API 20E system.

The type strain is UST050418-052^T (=JCM 13833^T =NRRL B-41465^T), isolated from the surface of the marine sponge *Halichondria panicea* collected from Friday Harbor, San Juan Island, WA, USA.

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