

***Roseovarius azorensis* sp. nov., isolated from seawater at Espalamaca, Azores**

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Abstract

A Gram-negative, motile, non-spore forming, rod shaped aerobic bacterium, designated strain SSW084^T, was isolated from a surface seawater sample collected at Espalamaca (38°33'N; 28°39'W), Azores. Growth was found to occur from 15 – 40 °C (optimum 30 °C), at pH 7.0 – 9.0 (optimum pH 7.0) and with 25 to 100 % seawater or 0.5 – 7.0 % NaCl in the presence of Mg²⁺ and Ca²⁺; no growth was found with NaCl alone. Colonies on seawater nutrient agar (SWNA) were observed to be punctiform, white, convex, circular, smooth, and translucent. Strain SSW084^T did not grow on Zobell Marine Agar (ZMA) and tryptic soy agar (TSA) even when seawater supplemented. The major respiratory quinone was found to be Q-10 and the G+C content was determined to be 61.9 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain SSW084^T belongs to the genus *Roseovarius* and that its closest neighbours are *Roseovarius tolerans* EL-172^T, *Roseovarius mucosus* DFL-24^T and *Roseovarius lutimaris* 112^T with 95.7, 95.4 and 95.3 % sequence similarity respectively. The remaining species of *Roseovarius* showed <95 % similarity. The polar lipids of strain SSW084^T were determined to be phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, an unidentified lipid and one unidentified aminolipid. The major fatty acids identified were identified as C_{18:1 ω7c} (52.5 %) and C_{16:0} (13.8 %). On the basis of phenotypic, molecular and chemotaxonomic characteristics, strain SSW084^T is considered to represent a novel species of the genus *Roseovarius*, for which *Roseovarius azorensis* sp. nov. is proposed. The type strain is SSW084^T (=KCTC 32421^T =MTCC 11812^T).

Keywords *Roseovarius azorensis* sp. nov · Polyphasic taxonomy · Seawater · Espalamaca

Introduction

The genus *Roseovarius*, a member of the family *Rhodobacteraceae* within the class *Alphaproteobacteria*, was first created by Labrenz *et al.* (1999) with a type strain, *Roseovarius tolerans* EL-172^T. The type species was characterized as Gram negative, rod-shaped, bacteriochlorophyll *a* (BChl *a*) positive, Q-10 as major quinone, C_{18:1} as major fatty acids and to require Na⁺ for growth. At the time of writing, the genus *Roseovarius* comprises 14 species with validly published names: *Roseovarius tolerans* (Labrenz *et al.* 1999), *Roseovarius nubinhibens* (González *et al.* 2003), *Roseovarius crassostreae* (Boettcher *et al.* 2005), *Roseovarius mucosus* (Biebl *et al.* 2005), *Roseovarius aestuarii* (Yoon *et al.* 2008), *Roseovarius halotolerans* (Oh *et al.* 2009), *Roseovarius pacificus* (Wang *et al.* 2009), *Roseovarius nanhaiticus* (Wang *et al.* 2010), *Roseovarius indicus* (Lai *et al.* 2011), *Roseovarius marinus* (Jung *et al.* 2011), *Roseovarius halocynthiae* (Kim *et al.* 2012b), *Roseovarius litoreus* (Jung *et al.* 2012), with recent proposal of *R. sediminilitoris* (Park and Yoon 2013) and *R. lutimaris* (Choi *et al.* 2013). In addition the species *Roseovarius marisflavi* has recently been proposed (Li *et al.* 2013). The members of the genus have been isolated from various aquatic environments, including seawater, sediment and lake. The main aim of the present study was to determine the precise taxonomic position of strain SSW084^T, which was isolated from Espalamaca, North Atlantic Ocean. This study uses a polyphasic approach that consists of determination of phenotypic and chemotaxonomic properties along with a detailed phylogenetic investigation based on 16S rRNA gene sequences. As a result of these studies, strain SSW084^T is considered to represent a novel species of the genus *Roseovarius*, for which *Roseovarius azorensis* sp. nov is proposed.

Materials and Methods

Bacterial strains and culture conditions

Surface seawater was collected from the Espalamaca region in the Azores, Portugal (38°33'N; 28°39'W) through an Indo-Portugal bilateral program during September 2010. Strain SSW084^T was isolated from a colony grown at 30 °C for 2 days on seawater nutrient agar (SWNA= nutrient agar prepared in 50 % seawater). Strain SSW084^T was purified by repeated streaking on SWNA. Subsequently, the growth of the strain SSW084^T was checked on tryptic soy agar (TSA), TSA prepared with 3 % NaCl, TSA prepared in 50 % seawater, nutrient agar prepared in artificial seawater (ASWNA) and Zobell marine agar (ZMA). Strain SSW084^T was maintained on SWNA at 4 °C for short-term maintenance and as 15 % glycerol suspensions at -80 °C for long-term preservation.

Morphological, physiological and biochemical characterization

Colony morphology (colour, shape, size, opacity, elevation) was observed using a colony counter. Gram staining (K001, Himedia) and motility by hanging drop (Collee *et al.* 2008) of strain SSW084^T was examined using light microscopy (Olympus BX-60) with 1000× magnification. The cell morphology was examined by scanning electron microscopy (JEOL JSM-5800LV). Salinity tolerance of strain SSW084^T was investigated in nutrient agar medium prepared in distilled water with NaCl concentrations of 0 – 15 % (w/v; 1 % increment) with and without adding Mg²⁺ and Ca²⁺ (4.53 g/L MgCl₂; 5.94 g/L MgSO₄; 1.3 g/L CaCl₂). In addition to this, strain SSW084^T was also tested in nutrient agar prepared with various seawater concentrations (0, 25, 50, 75 and 100 %). The pH range for growth was determined by adjusting the pH of the SWNB medium (nutrient broth prepared in 50 % seawater) using various buffers to pH 4–5 (citrate buffer), pH 6–8 (phosphate buffer) and pH 9–10 (carbonate buffer). Growth at various temperatures 4, 10, 15, 20, 30, 37, 40 and 45 °C was also determined by using SWNB medium. In above experiments, cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 2 days incubation.

For analysis of BChl *a*, strain SSW084^T was cultivated aerobically in the dark at 30 °C in SWNB medium. The cells were harvested by centrifugation at a speed of 10,000 rpm for 10 min. The cell pellet was used for extraction with methanol and the methanol solution was filtered in the dark. The filtered solution was examined for light absorbance spectrum from 400–900 nm with a spectrophotometer (Cary 300) to detect BChl *a* in the region of 766-772 nm (Shiba *et al.* 1979; Yoon *et al.* 2004).

Catalase activity was tested using the 3 % (v/v) H₂O₂ method (Smibert and Krieg 1994) and oxidase activity was determined using oxidase discs (DD018, Himedia, India). Hydrolysis of casein (1 %), gelatin (1 %), Tween 20 and 80 (1 %), tributyrin (1 %), xanthine (0.4 %) and alginate (1 %) (Smibert and Krieg 1994) were tested on SWNA medium. Hydrolysis of urea and DNA was determined on Urea agar base (M112, Himedia) and DNase test agar (M1041, Himedia) respectively. Xylan, starch and carboxymethyl cellulose (CMC) hydrolysis were determined using the methods provided by Khandeparker *et al.* (2011). Other enzyme activities were determined using API ZYM strips (BioMérieux) and acid production from carbohydrates was tested with API-CH-50 (BioMérieux) tests using inoculation medium API CHB/E (BioMérieux) amended with marine cations supplement (MCS, Farmer and Hickman-Brenner, 2006) according to the manufacturers' instructions. Antibiotic susceptibility tests were performed using antibiotic discs (Himedia) containing the following concentrations (µg per disc unless indicated): amikacin (30), amoxicillin (30), ampicillin (25), bacitracin (8 IU), cefadroxyl (30), ceftazidime (30), chloramphenicol (25), chlortetracycline (30),

ciprofloxacin (30), kanamycin (30), lincomycin (10), neomycin (30), norfloxacin (10), novobiocin (30), penicillin-G (2 IU), polymyxin-B (50 IU), rifampicin (15), streptomycin (300), tetracycline (10), tobramycin (10) and vancomycin (5).

Chemotaxonomic analysis

Cell biomass of strain SSW084^T for the analysis of isoprenoid quinones and polar lipids was obtained from cultures grown in LB broth (M1245, Himedia) prepared in 50 % seawater for 2 days at 30 °C. Analysis of whole cell fatty acids of the isolate was performed according to the Microbial Identification System TSBA 6.0 (Sasser 1990) after cultivation on SWNA for 3 days at 30 °C. Polar lipids were extracted and examined according to the procedures described by Collins *et al.* (1980). Briefly, Polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck). The first direction was developed in chloroform/methanol/water (65:25:3.8, by vol.) and the second in chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol.). Total lipid material and specific functional groups were detected using molybdophosphoric acid (total lipids), molybdenum blue spray reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α -glycols), and α -naphthol reagent (sugars). Isoprenoid quinones were determined as described by Minnikin *et al.* (1984). The genomic DNA for G+C content analysis was extracted using phenol method provided by Mesbah *et al.* (1989). The DNA G+C content of strain SSW084^T was determined by reverse-phase HPLC of nucleosides according to Mesbah *et al.* (1989).

Phylogenetic analyses

Genomic DNA of strain SSW084^T was isolated using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene was amplified using the bacterial consensus primers 27F and 1492R (Dojka *et al.* 1998). The PCR amplified 16S rRNA gene was purified with a PCR cleanup kit (Sigma) according to the manufacturer's instructions. DNA sequencing was performed using an automated 3130xl Genetic analyzer (Applied Biosystems). The sequence was aligned using Clustal W sequence alignment program (Thompson *et al.* 1994). The 16S rRNA gene sequences of the neighbouring taxa were obtained from the GenBank database. Phylogenetic trees were established with neighbour-joining (Saitou and Nei1987) and maximum-parsimony (Fitch 1971) algorithms using MEGA 5.0 software (Tamura *et al.* 2011) with bootstrap values based on 1,000 replications (Felsenstein 1985).

Results and Discussion

Cells of strain SSW084^T appeared as rod shaped, Gram-negative, approximately 0.6–0.8 µm wide and 1.3–2.0 µm long (Supplementary Fig. 1); Cells were found to be motile and non-spore forming. The colonies of the strain SSW084^T were observed to be punctiform, white, circular, convex, smooth and translucent on SWNA media after 48 h. Strain SSW084^T did not grow on medium with NaCl as a sole salt and was found to require 25 – 100 % seawater or 0.5 – 7.0 % NaCl with Mg²⁺ and Ca²⁺. Strain SSW084^T did not grow on marine agar and tryptic soy agar even when seawater supplemented. When tested on SWNB, growth of the strain SSW084^T was observed at temperatures between 15 and 40 °C with optimum growth at 30 °C, but no growth was observed at 10 and 45 °C. Growth of strain SSW084^T was found to occur at a pH range of 7.0–9.0 with an optimum pH of 7.0. Differences between morphological and physiological characteristics of strain SSW084^T compared with the closest related type strains of the genus *Roseovarius* are given in Table 1. Usually *Roseovarius* species are either pigmented or cream in colour and some of them are able to produce the pigment BChl *a*, whereas strain SSW084^T is non-pigmented and could not produce BChl *a* (data not shown). In addition strain SSW084^T was found to be susceptible to the following antibiotics: amikacin, amoxicillin, ampicillin, bacitracin, cefadroxyl, ceftazidime, chloramphenicol, chlortetracycline, ciprofloxacin, kanamycin, lincomycin, neomycin, norfloxacin, novobiocin, penicillin-G, polymyxin-B, rifampicin, streptomycin, tetracycline, tobramycin and vancomycin.

The 16S rRNA gene sequence obtained from the strain SSW084^T was found to be closely related to those of *R. tolerans* EL-172^T, *R. mucosus* DFL-24^T and *R. lutimaris* with sequence similarities of 95.72, 95.39 and 95.30 % respectively, which are below the threshold generally considered necessary of species delineation (Stackebrandt and Goebel 1994). Other related species of the genus *Roseovarius* showed <95 % similarity in EzTaxon-e (Kim *et al.* 2012a). The nearly full sequence (1,379 nucleotides) of the 16S rRNA gene of strain SSW084^T has been deposited in GenBank under accession number KC534172. Neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain SSW084^T formed a phylogenetic lineage with *R. tolerans* EL-172^T and *R. mucosus* DFL-24^T (bootstrap value 79; Fig. 1). The maximum-parsimony algorithm also confirmed the relationship of the strain SSW084^T with *R. tolerans* EL-172^T and *R. mucosus* DFL-24^T (Supplementary Fig. 2).

Strain SSW084^T was found to contain Q-10 as the major respiratory quinone, which is consistent with other *Roseovarius* species (Garrity and Holt 2001). The polar lipid profile of strain SSW084^T was found to consist of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, an unidentified lipid and one unidentified aminolipid (Supplementary Fig. 3).

The polar lipids are thus very similar to those of *R. mucosus* and *R. tolerans* (Labrenz *et al.* 1999; Biebl *et al.* 2005). The major fatty acids found in SSW084^T were C_{18:1} ω7c (52.5 %) and C_{16:0} (13.8 %), which are similar to the profiles of the type strains of *R. mucosus* and *R. tolerans* (Table 2). However, C_{18:2} which was reported as the second major fatty acid in *R. tolerans* was not detected in the strain SSW084^T. The genomic G+C content was found to be 61.9 mol% which is consistent with species of the genus *Roseovarius*.

In summary, from the phenotypic, genotypic and chemotaxonomic data presented above it can be concluded that the strain SSW084^T is a member of the genus *Roseovarius* but can be distinguished from previously described species of the genus (Table 1). Strain SSW084^T therefore represents a novel *Roseovarius* species, for which we proposed the name *Roseovarius azorensis* sp. nov.

Description of *Roseovarius azorensis* sp. nov

Roseovarius azorensis (*azo.ren'sis*, N.L. masc. adj. *azorensis*, after the portug. Islas dos Azores, pertaining to the Azorean Islands, the source of the sample from which the type strain was isolated).

Gram-negative, rod shaped (0.6–0.8 × 1.3–2.0 μm), motile by gliding and non-spore forming. The colonies are punctiform, non-pigmented, circular, convex, smooth and translucent on SWNA medium. Requires 25 – 100 % seawater for its growth or can grow with salinity range of 0.5 – 7.0 % NaCl in the presence of Mg²⁺ and Ca²⁺; does not grow with NaCl alone. Growth is observed at temperature ranges from 15–40 °C and at pH 7.0–9.0. The optimum temperature and pH for growth are 30 °C and 7.0 respectively. BChl *a* is not produced. Oxidase positive but catalase negative. Nitrate reduction is positive. Acid production is observed from the substrate potassium 5-keto gluconate but not from other carbohydrates in the API-CH-50 gallery. Aesculin is hydrolyzed but starch, DNA, gelatin, Tween 20 and 80, casein, tributyrin, xanthine, alginate, urea, xylan and CMC are not hydrolysed. Positive (in API ZYM strips) for esterase C4, esterase lipase C8, lucine arylamidase and naphthol-AS-BI-phosphohydrolase but negative for alkaline phosphatase, trypsin, α-glucosidase, β-glucosidase, acid phosphatase, lipase C14, valine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase, N-acetyl-β-glucosaminidase, α-chymotrypsin. The polar lipids are phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, an unidentified lipid and one unidentified aminolipid. The predominant ubiquinone is Q-10. The major fatty acids are C_{18:1} ω7c and C_{16:0}. The G+C content of the type strain is 61.9 mol%.

The type strain, SSW084^T (= KCTC 32421^T = MTCC 11812^T), was isolated from surface seawater at Espalamarca, Azores (North Atlantic Ocean), Portugal. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain SSW084^T is KC534172.

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Table 1 Characteristic that differentiate strain SSW084^T and the closely related type strains of the genus *Roseovarius*

Data for *R. tolerans*, *R. mucosus*, *R. lutimaris* and *R. aestuarii* are taken from Labrenz *et al.* (1999), Biebl *et al.* (2005), Choi *et al.* (2013) and Yoon *et al.* (2008) respectively. All strains are positive for esterase (C4) and esterase lipase (C8). All strains are negative for citrate utilization, indole production, hydrolysis of starch, Tween 20 & 80, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. +, positive; -, negative; W, weak; ND, not determined; *Data for *R. tolerans*, *R. mucosus*, *R. lutimaris* taken from Choi *et al.* (2013).

Characteristic	SSW084 ^T	<i>R. tolerans</i>	<i>R. mucosus</i>	<i>R. lutimaris</i>	<i>R. aestuarii</i>
Pigmentation	Non-pigmented	Red	Faintly pink	Beige	Cream
Cell morphology	Rod	Rod	Rod	Ovoid/Rod	Ovoid/Rod
Cell size	0.6–0.8×1.3–2.0	0.7–1.0×1.1–2.2	0.5–0.7×1.3–3.0	0.4–0.8×2.0–3.0	0.3–0.6×0.8–3.5
Motility	+	+	-	+	+
Catalase	-	+	+	+	+
Oxidase	+	W	+	+	+
Growth ranges					
pH	7.0–9.0	5.9–9.0	6.0–8.8	5.0–9.0	7.0–8.0
Temperature (°C)	15–40	8–33.5	20–40	15–40	10–37
NaCl (%)	0.5–7.0	1.0–10.0	1.0–7.0	1.0–6.0	0.5–7.0
BChl <i>a</i>	-	+	+	-	-
Nitrate reduction	+	-	-	+	+
Gelatin liquefaction	-	-	+	-	-
Enzyme activities*					

Alkaline phosphatase	-	+	+	+	W
Valine arylamidase	-	+	+	W	-
Cystine arylamidase	-	+	W	-	-
Trypsin	-	W	-	-	-
Acid phosphatase	-	+	+	-	-
Acid production from*					
D-Arabinose	-	+	+	+	ND
D-Galactose	-	+	+	+	-
D-Glucose	-	-	+	W	-
D-Fructose	-	+	+	+	-
D-Mannitol	-	-	+	-	-
D-Sorbitol	-	-	+	W	-
DNA G+C content (mol %)	61.9	62.2–63.8	60.9–62.9	58.2	58.6
Isolation source	Sea water	Lake water	Dinoflagellate	Tidal flat	Tidal flat

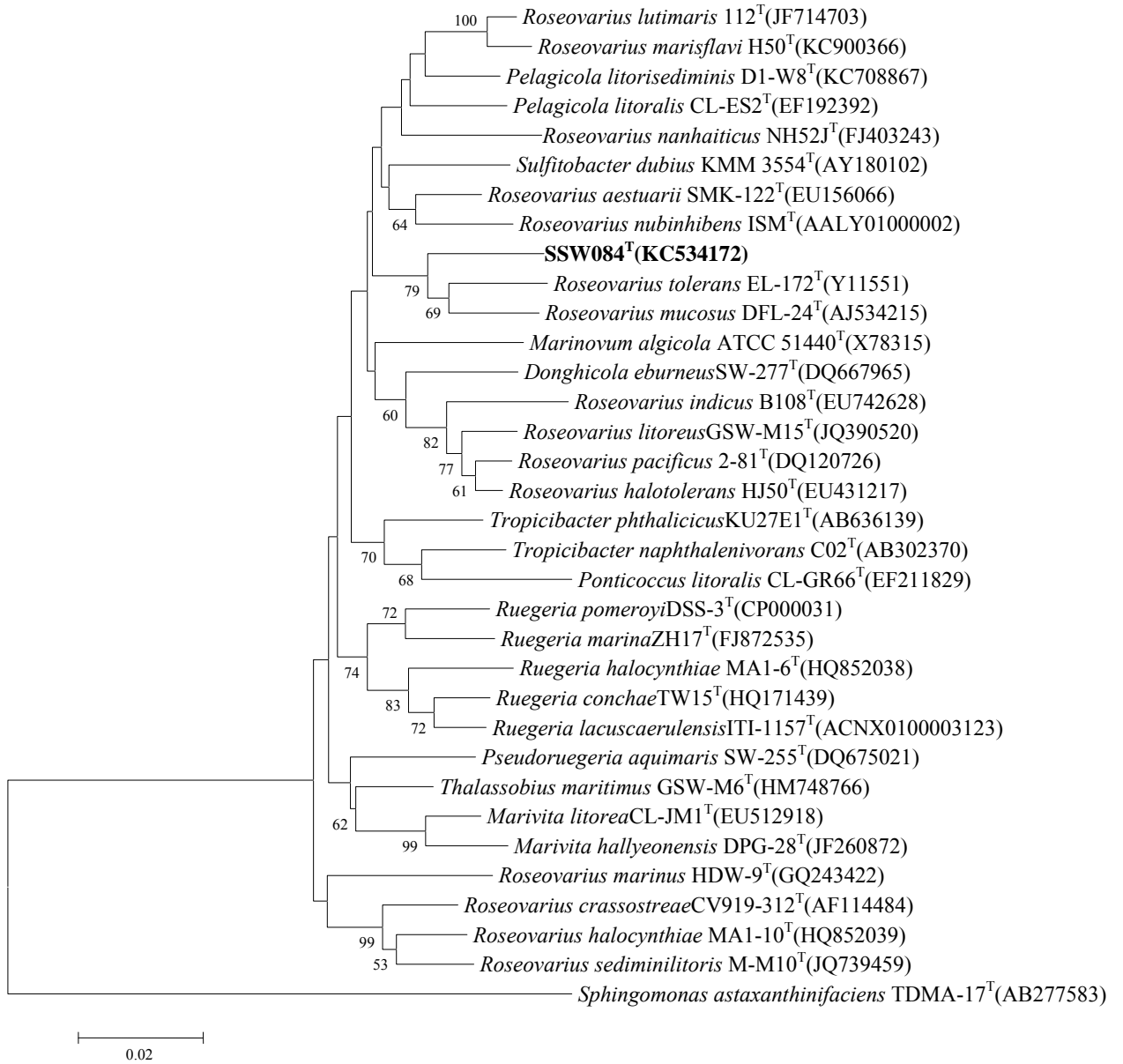
Table 2. Fatty acid composition of strain SSW084^T and the closest related type strains *R. tolerans* and *R. mucosus*.

Data for *R. tolerans* and *R. mucosus* are taken from Labrenz *et al.* (1999) and Biebl *et al.* (2005) respectively.

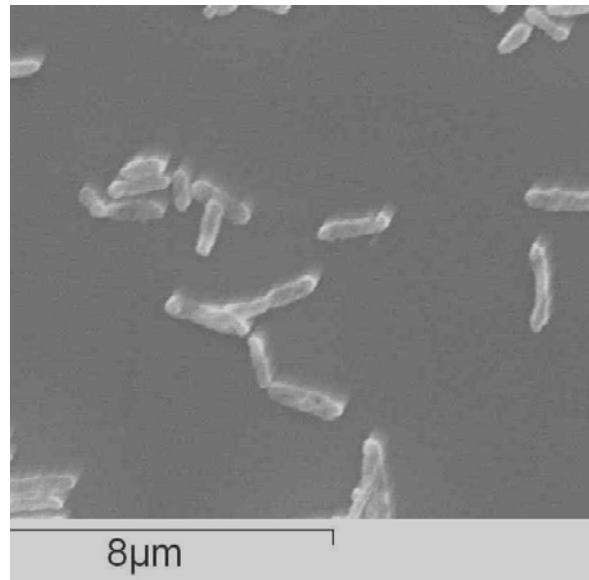
Fatty acids	SSW084 ^T	<i>R. tolerans</i>	<i>R. mucosus</i>
C _{12:0}	4.5	-	3.1
C _{16:0}	13.8	6.2	13.1
C _{18:0}	4.1	0.8	1.8
C _{18:2}	-	10.6	-
C _{12:0} 2-OH	-	2.4	2.5
C _{12:0} 3-OH	3.8	-	-
C _{12:1} 3-OH	-	3.6	1.7
iso-C _{11:0} 3-OH	8.3	-	-
iso-C _{18:1} H	1.3	-	-
C _{18:1} ω7 <i>c</i>	52.5	70.2	69.4
Methyl-C _{18:0}	-	-	2.1
Methyl-C _{18:1} ω7 <i>c</i>	1.7	-	2.1
C _{18:1} ω9 <i>c</i>	2.4	-	-
Cyclo-C _{19:0} ω8 <i>c</i>	2.1	-	-
Cyclo-C _{19:1}	-	-	3.7
C _{16:1} ω6 <i>c</i> / C _{16:1} ω7 <i>c</i>	1.9	-	-

Legends to figures

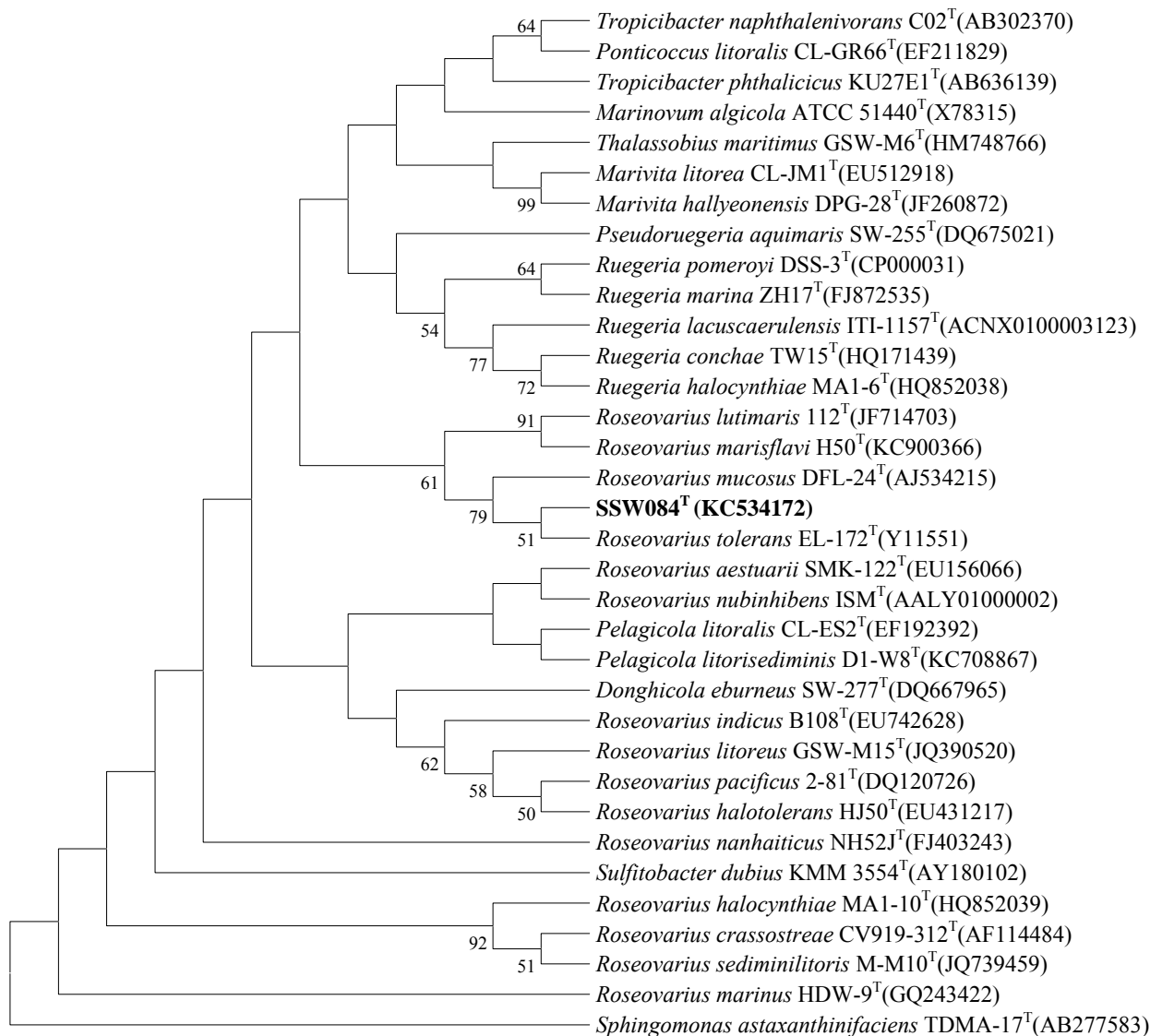
Fig. 1 Neighbour-joining tree showing the phylogenetic relationship of strain SSW084^T and phylogenetically related species of the family *Rhodobacteraceae* based on 16S rRNA gene sequences. Bootstrap percentages (1,000 replications) are shown at branching points and the values >50 % are shown. *Sphingomonas astaxanthinifaciens* TDMA-17^T (AB277583) was used as an out group. Scale bar 0.02 substitutions per nucleotide position.



Supplementary Fig. 1 Scanning electron micrograph of strain SSW084^T after growth for 3 days at 30 °C on SWNA



Supplementary Fig. 2 Maximum-parsimony tree showing the phylogenetic relationship of strain SSW084^T and phylogenetically related species of the family *Rhodobacteraceae* based on 16S rRNA gene sequences



Supplementary Fig. 3 Two-dimensional TLC of total polar lipids of the strain SSW084^T. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; AL, unidentified aminolipid; DPG, diphosphatidylglycerol; L, unidentified lipid

