Roseovarius nanhaiticus sp. nov., a member of the *Roseobacter* clade isolated from marine sediment

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An aerobic, Gram-staining-negative, rod or ovoid-shaped bacterial isolate, strain NH52J^T, was isolated from a sandy sediment sample from the South China Sea. Strain NH52J^T exhibited tumbling motility, formed beige or faint pink colonies, gave a positive reaction in tests for catalase and oxidase and required NaCl for growth. Optimal growth was observed at pH 7.8–9.3, at 30 °C and in the presence of 2.0–4.0 % (w/v) NaCl. The novel strain did not synthesize bacteriochlorophyll *a*, and the DNA G+C content was 62 %. The major fatty acids were C_{18:1}ω7c, C_{16:0} and C_{18:1}ω7c 11-methyl. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NH52J^T was affiliated to the genus *Roseovarius* of the class *Alphaproteobacteria*. *Roseovarius pacificus* and *Roseovarius aestuarii* were the most closely related recognized species to strain NH52J^T with 16S rRNA gene sequence similarity values of 95.0 and 95.7 %, respectively. Sequence similarity values between strain NH52J^T and other phylogenetically related species were all below 95.0 %. Based on the phenotypic, chemotaxonomic and phylogenetic data presented, strain NH52J^T is considered to represent a novel species of the genus *Roseovarius*, for which the name *Roseovarius nanhaiticus* sp. nov. is proposed. The type strain is NH52J^T (=LMG 24840^T=CCTCC AB 208317^T=MCCC 1A03543^T).

The genus *Roseovarius* comprised six recognized species at the time of writing: *Roseovarius tolerans* (Labrenz *et al.*, 1999), *Roseovarius nubinihibens* (González *et al.*, 2003), *Roseovarius crassostreae* (Boettcher *et al.*, 2005), *Roseovarius mucosus* (Biebl *et al.*, 2005), *Roseovarius aestuarii* (Yoon *et al.*, 2008) and *Roseovarius pacificus* (Wang *et al.*, 2009). The genus *Roseovarius* accommodates Gram-negative, ovoid or rod-shaped, obligate aerobic alphaproteobacteria and all its members are positive in tests for catalase. All of the species were retrieved from marine environments and belong to the *Roseobacter* group. Many novel representatives of this group have been isolated from marine habitats all over the world and described recently (Wagner-Döbler *et al.*, 2003; Buchan *et al.*, 2005; Dai *et al.*, 2006; Lee *et al.*, 2007; Hwang & Cho, 2008). In this paper, a novel Gram-negative, beige- to slightly pink-pigmented, aerobic bacterium is described that was isolated from sediment of the South China Sea. By using 16S rRNA gene sequence phylogenetic analysis, the new strain was determined to be a member of the *Roseobacter* group, in which it formed a distinct lineage

with members of the genus *Roseovarius*. Based on differences in fatty acid composition, physiological and biochemical characteristics, morphological features, and phylogenetic analysis as described in this study, the new strain should be classified as a novel species of the genus *Roseovarius*.

Recently, bacterial diversity investigations have led to the discovery of many novel species and genera from the South China Sea. One of these strains, designated NH52J^T, was isolated from a brown sandy sediment sample (109° 40.102' E, 06° 5.901' N), which was collected at water depth of 157 m in June 2007. In the laboratory, the sediment sample was suspended and diluted (1 : 100) using sterilized seawater, spread onto M2 agar plates and then incubated at 25 °C for 1 week. The composition of M2 medium was (l⁻¹ seawater): 5 g CH₃COONa, 0.5 g peptone, 0.5 g yeast extract, 0.5 g glucose, 0.5 g sucrose, 0.5 g starch, 0.05 g trisodium citrate, 0.05 g malic acid, 0.05 g potassium sodium tartrate, 1.0 g NH₄NO₃, 0.2 g NH₄Cl and 15 g agar, adjusted to pH 7.5–7.6. Strain NH52J^T was picked and streaked on fresh M2 agar medium. This process was repeated three times for purification and the strain was preserved as a 20 % (v/v) glycerol suspension at –80 °C. Cell morphology and motility were observed by phase-contrast microscopy

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Abbreviation: Bchl *a*, bacteriochlorophyll *a*.

The GenBank accession number for the 16S rRNA gene sequence of *Roseovarius nanhaiticus* sp. nov. NH52J^T is FJ403243.

Table 1. Phenotypic characteristics that differentiate strain NH52J^T from the closely related recognized species of the *Roseobacter* clade

Strains: 1, NH52J^T; 2, *Rva. aestuarii* SMK-122^T; 3, *Rva. pacificus* 81-2^T; 4, *Rva. tolerans* DSM 11457^T; 5, *Rva. nubinihibens* ISM^T; 6, *Rva. crassostreae* CV919-312^T; 7, *Rva. mucosus* DFL-24^T; 8, *Leisingera methylohalidivorans* MB2^T; 9, *Phaeobacter gallaeciensis* BS107^T; 10, *Marinovum algicola* ATCC 51440^T. Data for reference type strains are from Yoon *et al.* (2008), Biebl *et al.* (2005), Labrenz *et al.* (1999), González *et al.* (2003), Boettcher *et al.* (2005), Martínez-Cánovas *et al.* (2004), Martens *et al.* (2006), Schaefer *et al.* (2002), Ruiz-Ponte *et al.* (1998), Lafay *et al.* (1995) and this study. All strains were positive in tests for catalase and all strains have a requirement for Na⁺ ions for growth. +, Positive; −, negative; w, weakly positive; v, variable; ND, no data available; NF, not found.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell shape	Rods	Ovoid to rods	Rod to ovoid	Short rods	Short rods	Rod to ovoid	Short rods	Rods or ovoid rods	Ovoid rods	Ovoid
Source of isolation	South China Sea	Yellow Sea tidal flat	Sediment (West Pacific Ocean)	Hypersaline (Antarctic lake)	Seawater (Caribbean Sea)	Animal (<i>Crassostrea virginica</i>)	Marine dinoflagellates	Seawater (coast of California)	Seawater	Marine dinoflagellate
Colony colour	Beige/pink	Cream	Faintly pink	Red	Cream	Pink-beige	Whitish to faintly pink	—	Brown	Beige to pink
Motility	+	+	+	+	+	+	—	+	+	+
Flagella	NF	Polar	Subpolar	NF	ND	Subpolar to lateral	NF	Subpolar	Polar	Subpolar
Catalase	+	+	+	+	+	+	+	+	+	+
Bchl <i>a</i>	—	—	—	+	ND	—	tr	—	—	—
Salinity range (% w/v)	0.5–10.0	≤7	1–15	1–15	0.8–4.7	ND	0.3–10	1–6	0.6–11.7	0.5–12
Optimal salinity for growth (%)	2.0–4.0	ND	2–12	1–8	1.1–2.3	1–1.5	1–7	3	1.2	ND
Optimum temperature (°C)	30	30	25	8.5–33.5	30	34	31	27	23–27	25–30
Growth at										
4 °C	—	—	w	+	—	—	—	+	—	—
37 °C	+	+	+	+	+	+	+	—	+	+
pH optimum	7.8–9.3	7.0–8.0	6.2–8.5	6.2–9.0	ND	6.5–8.0	6.5–8.8	7.7	7.0	7.5
Hydrolysis of:										
Aesculin	—	—	—	—	—	—	—	ND	ND	+
Gelatin	—	—	—	—	—	—	—	—	—	+
Starch	—	—	—	—	—	—	—	—	—	+
Casein	—	+	ND	—	—	—	—	ND	ND	ND
Tween 20	—	—	—	—	w	—	+	ND	ND	—
Tween 40	—	—	w	—	—	w	+	ND	ND	+
Nitrate to nitrite	—	+	—	—	—	+	—	—	—	—
Enzyme activities:										
β-Galactosidase	—	—	—	—	—	—	—	ND	—	+
Alkaline phosphatase	w	w	ND	+	+	+	—	ND	ND	—
Leucine arylamidase	+	—	ND	+	+	+	+	ND	ND	ND

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10
Acid production from:										
D-Mannitol	–	–	–	+	–	–	–	ND	ND	–
Carbon source:										
Citrate	–	–	W	–	–	–	+	–	–	+
Glucose	–	–	–	–	+	–	–	–	+	+
Fructose	–	–	–	–	–	–	–	–	+	+
L-Malate	–	+	+	+	–	+	+	–	ND	+
Susceptible to:										
Penicillin G	+	+	+	+	+	+	+	+	–	+
Tetracycline	–	–	+	+	–	–	–	ND	ND	W
Neomycin	–	+	+	+	+	+	+	ND	ND	–
DNA G+C content (mol%)	62	59	62	63	66	59	61–63	60	58	64–65

(model 50i; Nikon) and transmission electron microscopy (model JEM-1230; JEOL) using cells from the early exponential phase grown at 30 °C for 2 days.

The temperature range for growth was determined between 4 and 45 °C. The initial growth pH range (3–10) was determined using 5 ml HLB medium supplemented with 200 µl M2 medium, adjusted with NaOH (5 M) and HCl (5 M) solution. HLB medium was modified from Luria–Bertani (LB) medium (Sambrook *et al.*, 1989), with the concentration of NaCl increased to 30 g l^{–1}. The salinity range supporting growth was determined at various NaCl concentrations (0.5–25 %, w/v) in M2 medium. The requirement for NaCl was determined in M2 medium lacking Na⁺. The salinity and pH range experiments were conducted with an incubation temperature of 30 °C. Growth was measured by using a spectrophotometer at 600 nm after incubation for 2 days. Gram-staining was performed as described by Gerhardt *et al.* (1994). The oxidase reaction was tested by using oxidase reagent (bioMérieux). Catalase activity was tested using a 3 % H₂O₂ solution. Hydrolysis of starch, casein, Tween 20, 40 and 80 were determined as described by Cowan & Steel (1965). Other physiological and biochemical tests were performed using API 20NE, API 20E and API ZYM strips (bioMérieux). The inoculum was prepared by suspending cells in a 3 % (w/v) NaCl solution and the salinity of the API AUX medium was also increased to 3 %. Production of bacteriochlorophyll *a* (Bchl *a*) was determined by spectrophotometric analysis as described by Martens *et al.* (2006). The results are presented in Table 1.

Susceptibility to antibiotics was tested on M2 agar plates by using the following discs (Oxoid; µg per disc unless otherwise specified): ceftriaxone (30), cephradine (30), chloramphenicol (30), gentamicin (10), erythromycin (15), cefoperazone (75), ciprofloxacin (5), clindamycin (2), doxycycline hydrochloride (30), neomycin (10), tetracycline (30), cephalixin (30), ampicillin (10), furazolidone (15), metronidazole (5), cephalozin (30), lincomycin (2), minocycline (30), norfloxacin (10), kanamycin (30), vancomycin (30), trimethoprim (25), piperacillin (100), ofloxacin (5), rifampicin (5), carbenicillin (100), polymyxin B (300 U), streptomycin (10), oxacillin (1) and penicillin G (10 U). All of the test plates were incubated at 30 °C for 2 days.

For cellular fatty acid analysis, strain NH52J^T was harvested from MA plates after cultivation for 3 days at 30 °C. The fatty acids were extracted according to the standard protocol of the Microbial Identification System (MIDI, Sherlock). Analysis of the fatty acid methyl esters was performed by GC (6850, Agilent) and peaks were identified with MIDI software (version 6.0). The predominant cellular fatty acids of strain NH52J^T were straight-chain unsaturated and saturated fatty acids, methylated fatty acids and hydroxy fatty acids. Fatty acids that constituted >5 % of the total were C_{18:1}ω7c (50.4 %), C_{16:0} (18.6 %), C_{12:0} (9.2 %), C_{18:1}ω7c 11-methyl (9.3 %) and C_{12:0} 3-OH

(5.3%). A relatively large amount of $C_{18:1\omega7c}$ is one feature found for the majority of species within the class *Alphaproteobacteria* (Martens *et al.*, 2006). The major fatty acid profile was similar to those of recognized species of the genus *Roseovarius* (Table 2).

The genomic DNA of strain NH52J^T was extracted according to the method described by Ausubel *et al.*

Table 2. Comparison of the cellular fatty acid contents (%) of strain NH52J^T with recognized species of the genus *Roseovarius*

Strains: 1, NH52J^T (data from this study); 2, *Rva. aestuarii* SMK-122^T; 3, *Rva. tolerans* DSM 11457^T; 4, *Rva. nubinihibens* DSM 15170^T; 5, *Rva. crassostreae* CV919-312^T; 6, *Rva. mucosus* DFL-24^T. Data for reference type strains are from Yoon *et al.* (2008). Cell mass of strain NH52J^T and for the type strains of the five species of the genus *Roseovarius* was harvested from MA plates after cultivation for 3 days at 30 °C (Yoon *et al.*, 2008). ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5	6
Straight-chain saturated fatty acids						
$C_{12:0}$	9.2	5.1		5.1		6.6
$C_{14:0}$	0.5					
$C_{15:0}$		0.2	1.9	0.6		
$C_{16:0}$	18.6	16.2	10.3	13.8	11.8	8.1
$C_{17:0}$		0.3	5.8	0.6	0.3	0.3
$C_{18:0}$	0.7	1.0	1.2	0.9	1.5	1.0
Straight-chain unsaturated fatty acids						
$C_{17:1\omega8c}$		0.2	1.0			
$C_{18:1\omega7c}$	50.4	68.0	67.4	58.5	77.9	65.1
Branched-chain saturated fatty acids						
anteiso- $C_{15:0}$	0.5					
anteiso- $C_{16:0}$	0.5					
anteiso- $C_{17:0}$	0.5					
Hydroxy fatty acids						
$C_{10:0}$ 3-OH	0.3	0.2			2.0	1.0
$C_{12:0}$ 2-OH			0.6			1.5
$C_{12:0}$ 3-OH	5.3	5.3		2.3	0.1	0.3
$C_{12:1}$ 3-OH			2.6			2.8
$C_{16:0}$ 2-OH			0.9			4.6
$C_{16:0}$ 3-OH					0.6	
Methyl fatty acids						
$C_{18:1\omega7c}$ 11-methyl	9.3	0.8	6.1		1.5	0.9
Cyclopropane acid						
$C_{19:0\omega8c}$ cyclo	0.6	1.5	0.2	14.2		4.3
Summed features*						
3	1.6	0.6	0.6	1.5	2.2	1.0
7	0.5		0.3			1.3
Unknown fatty acid (ECL 11.799)	0.9	0.2		0.6		0.9

*Summed features represent groups of two or three fatty acids that could not be separated by the MIDI system. Summed feature 3 contains $C_{16:1\omega7c}$ and/or iso- $C_{15:0}$ 2-OH. Summed feature 7 contains $C_{19:1\omega6c}$, $C_{19:0\omega10c}$ cyclo and/or unknown fatty acid ECL 18.846.

(1995). The 16S rRNA gene was PCR-amplified with two universal primers, 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-ACGGCTACCTTGTACGACT-3'. Purification and sequencing of PCR products were performed as described by Liu & Shao (2005). A nearly full-length 16S rRNA gene sequence (1422 bp) of strain NH52J^T was obtained and was compared with sequences retrieved from the GenBank database. Sequences were aligned using DNAMAN software (version 6) and evolutionary distances were calculated according to the Kimura two-parameter model analysis (Kimura, 1980). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and evaluated by bootstrap analysis based on 1000 replicates.

The DNA G + C content was determined to be 62 mol% by reverse HPLC according to the method of Tamaoka & Komagata (1984). This value is consistent with the G + C range of the genus *Roseovarius* (58.6–66 mol%).

Comparative 16S rRNA gene sequence analysis showed that strain NH52J^T was most closely related to the members of the genera *Roseovarius*, *Leisingera*, *Phaeobacter* and *Marinovum* within the class *Alphaproteobacteria*. Strain NH52J^T exhibited 16S rRNA gene sequence similarity values of 95.2, 95.7, 94.7, 94.6, 93.9, 93.6, 94.0, 93.7 and 93.1 % with respect to the type strains of *Roseovarius pacificus*, *Roseovarius aestuarii*, *Roseovarius mucosus*, *Roseovarius nubinihibens*, *Roseovarius crassostreae*, *Roseovarius tolerans*, *Leisingera methylohalidivorans*, *Marinovum algicola* and *Phaeobacter gallaeciensis*, respectively. Moreover, the 16S rRNA sequence similarities with other species were less than 94.0%. A phylogenetic tree constructed by using the neighbour-joining algorithm indicated that strain NH52J^T was unambiguously affiliated to the genus *Roseovarius* in a distinct clade. Within this clade, strain NH52J^T and the following species *Rva. pacificus*, *Rva. aestuarii*, *Rva. nubinihibens* and *Rva. crassostreae* formed a clearly separate subclade, which was grouped together with other species of the genus *Roseovarius* (Fig. 1). The results of the phylogenetic analysis and the relatively low 16S rRNA gene sequence similarity to species of the genus *Roseovarius* suggest that strain NH52J^T represents a novel species within the genus *Roseovarius*.

The hydroxylated fatty acids $C_{12:0}$ 2-OH, $C_{12:0}$ 3-OH and $C_{12:1}$ 3-OH have been indicated as being characteristic of the genus *Roseovarius* (Biebl *et al.*, 2005). Fatty acid $C_{12:0}$ 3-OH was present in strain NH52J^T and the following four strains, *Rva. aestuarii*, *Rva. nubinihibens*, *Rva. crassostreae* and *Rva. mucosus*, whereas *Rva. tolerans* contained fatty acids $C_{12:1}$ 3-OH and $C_{12:0}$ 2-OH instead. For *Rva. mucosus*, hydroxylated fatty acids $C_{12:0}$ 2-OH, $C_{12:0}$ 3-OH and $C_{12:1}$ 3-OH were all detected. The very similar major fatty acid composition also supported the placement of the novel isolate within the genus *Roseovarius* (Table 2). Strain NH52J^T was distinguishable from other species of the genus *Roseovarius* by fatty acid $C_{17:0}$, which was absent in this strain, and the presence of branched-chain fatty acids

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of strain NH52J^T and representative members of the family *Rhodobacteraceae* within the class *Alphaproteobacteria*. The tree was generated by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values above 50 % (1000 replicates) are indicated at the nodes. GenBank accession numbers are shown in parentheses. Bar, 0.05 substitutions per nucleotide position.

that were only found in the novel isolate, such as anteiso-C_{15:0}, anteiso-C_{16:0} and anteiso-C_{17:0}. The fatty acid data, as well as the results of phylogenetic analyses, support the classification of strain NH52J^T as a novel species of the genus *Roseovarius*.

The phenotypic characteristics of strain NH52J^T are given in the species description below. Growth occurred at NaCl concentrations of 0.5–10.0 % (w/v), but was not observed without NaCl or at NaCl concentrations higher than 13.0 % (w/v). Optimal growth occurred at 2.0–4.0 % (w/v) NaCl. Within the tested temperature range (4–45 °C), growth occurred between 9 °C and 37 °C, with maximum growth at 30 °C. The optimal pH for growth was 7.8–9.3; weak growth occurred at pH 6.5 and 9.7, but no growth occurred at pH 6.0 or 10.0. Other differences in phenotypic characteristics between

strain NH52J^T and some phylogenetically related members of the *Roseobacter* clade are shown in Table 1. The low 16S rRNA gene sequence similarity, together with differences found in chemotaxonomic, physiological and biochemical analyses, indicates that isolate NH52J^T should be classified in the genus *Roseovarius* as a novel species. The name *Roseovarius nanhaiticus* sp. nov. is proposed.

Description of *Roseovarius nanhaiticus* sp. nov.

Roseovarius nanhaiticus (nan.hai'ti.cus. N.L. masc. adj. *nanhaiticus* referring to Nanhai, the South China Sea, the site from where the type strain was isolated).

Cells are aerobic, Gram-staining-negative, non-spore-forming and ovoid to rods with a size of 0.58–0.84 × 1.42–3.37 µm (Fig. 2). Cells show predominantly

Fig. 2. Morphology of cells of strain NH52J^T grown on an M2 agar plate for 24 h at 30 °C. (a) Phase-contrast micrograph; bar, 10 µm. (b–c) transmission electron micrographs of negatively stained cells; bars, 1 µm.

tumbling motion. Flagella are not found. On M2 agar plates, small, wettish, smooth, beige or faint pink colonies develop that are 0.5–1.0 mm in diameter within 3–5 days incubation at 30 °C. Optimal growth is observed at 30 °C, at salinities of 2.0–4.0 ‰ and at pH values between 7.8 and 9.3. There is an absolute requirement for sodium ions. Bacteriochlorophyll *a* is not detected. Oxidase and catalase reactions are positive. Nitrate reduction, hydrolysis of starch, Tween 20, 40 and 80, gelatin liquefaction and urease activity are negative. H₂S and indole are not produced. Voges–Proskauer reaction is positive. Tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, β-galactosidase and tryptophan deaminase are negative. In assays with API ZYM strips, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and cystine arylamidase are positive, but lipase (C14), trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. Cells are fastidious and grow well on seawater-based complex medium, i.e. with yeast extract, tryptone and potato peptone as the substrates. According to the API 20NE strip, D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, citrate and phenylacetic acid are not utilized as sole carbon sources. Acid is not produced from inositol, D-sorbitol, L-rhamnose, sucrose, melibiose or

amygdalin in the API 20E system. In addition, the type strain is susceptible to cefoperazone, ciprofloxacin, ceftriaxone, erythromycin, cephalexin, ampicillin, kanamycin, norfloxacin, cephazolin, minocycline, carbenicillin, streptomycin, ofloxacin, rifampicin, piperacillin, penicillin G, chloramphenicol, cephadrine and gentamicin, but resistant to polymyxin B, clindamycin, lincomycin, furazolidone, doxycycline hydrochloride, tetracycline, metronidazole, vancomycin, trimethoprim, oxacillin and neomycin. The predominant fatty acids are (>5 % of total fatty acids): C_{18:1}ω7c, C_{16:0}, C_{12:0}, C_{18:1}ω7c 11-methyl and C_{12:0} 3-OH. Other phenotypic characteristics are given in Table 1.

The type strain, NH52J^T (=LMG 24840^T=CCTCC AB 208317^T=MCCC 1A03543^T), was isolated from a sandy sediment sample of the South China Sea at a depth of 157 m. The DNA G+C content of the type strain is 62 mol%.

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