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Leisingera nanhaiensis sp. nov., isolated from marine sediment of the South China Sea

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The GenBank accession number for the 16S rRNA gene sequence of *Leisingera nanhaiensis* NH52F^T is FJ232451.

A table comparing the cellular fatty acid content of strain NH52F^T with that of other related recognized species is available as supplementary data in IJSEM Online.

Summary: An aerobic, Gram-staining-negative, motile, rod-shaped bacterium, strain NH52F^T, was isolated from a sandy sediment sample in the South China Sea. On M2 agar medium (a complex medium), colonies were beige in colour. The isolate showed highest 16S rRNA gene sequence similarities to members of the genera *Leisingera* (96.7% similarity), *Phaeobacter* (95.4-96.0%) and *Marinovum* (94.1%). Phylogenetic analysis based on 16S rRNA gene sequence indicated that strain NH52F^T formed a distinct cluster with *Leisingera methylohalidivorans* MB2^T and *Leisingera aquimarina* LMG 24366^T. Optimal growth was observed at pH 7.0-8.5 and 25°C and requires the presence of 1-4% (w/v) NaCl. The major fatty acids were $C_{18:1}\omega7c$, $C_{16:0}$ 2-OH, $C_{10:0}$ 3-OH, $C_{12:0}$ 3-OH, $C_{16:0}$ and 11-methyl $C_{18:1}\omega7c$. The DNA G+C content was 60.5%. The phylogenetic and chemotaxonomic characteristics of strain NH52F^T were similar to those of the genus *Leisingera*. However, the differences in phenotypic properties and the similarity value between 16S rRNA gene, demonstrated the isolate differs from the recognized *Leisingera* species. On the basis of phenotypic, chemotaxonomic and phylogenetic data, this organism should be classified as a representative of a novel species in the genus *Leisingera*, for which the name *Leisingera nanhaiensis* sp. nov. is proposed. The type strain is NH52F^T (=LMG 24841^T=CCTCC AB 208316^T=MCCC 1A04178^T).

The genus *Leisingera* was proposed by Schaefer *et al.* (2002) for accommodating Gram-stain-negative, aerobic, rod-shaped marine bacteria which are able to grow on methyl halides as a sole carbon source. Recently, the emended description of the genus *Leisingera* was given by Martens *et al.* (2006) and Vandecandelaere *et al.* (2008), with additional chemotaxonomic data and some physiological and biochemical features involved. To data, the

genus includes two species of marine bacteria, *Leisingera methylohalidivorans* and *Leisingera aquimarina* (Schaefer *et al.*, 2002; Vandecandelaere *et al.*, 2008). The former species was isolated from a tide pool off the coast of California. The latter species was isolated from a marine electroactive biofilm grown on a stainless steel cathode (Genoa, Italy). Comparative 16S rRNA gene sequence analysis has shown that the genus *Leisingera* is phylogenetically closely related to the genera *Marinovum* and *Phaeobacter* within the *Roseobacter* clade (Schaefer *et al.*, 2002; Martens *et al.*, 2006). Here we describe a bacterial strain, NH52F^T as the type strain of a novel species of this genus on the base of physiological and biochemical characterization, chemotaxonomic properties and phylogenetic analysis of the 16S rRNA gene sequences.

Strain NH52F^T was isolated from a brown sandy sediment sample of the South China Sea (109° 40.102´ E, 06° 5.901´ N), which was collected from a water depth of 157 m. In the laboratory, the sediment was suspended and diluted (1:100) using the sterilized seawater, and then spread onto M2 agar plates, incubated at 25 °C for 1 week. Composition of the medium (M2) was (l^{-1} sea water): 5 g CH_3COONa, 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 NH_4NO_3, 0.2 g NH_4Cl and 15 g agar, adjusted to pH 7.5~7.6. Strain NH52F^T was picked out and streaked for purity three times on fresh M2 agar medium and preserved as a 20% (v/v) glycerol suspension at -80 °C. Cell morphology and motility was observed by phase-contrast microscopy (model 50i; Nikon) and transmission electron microscopy (model JEM-1230; JEOL), using cells from the early exponential phase grown on M2 agar.

Cells of strain NH52F^T were Gram-staining-negative, motile rod-shaped and 0.62-0.8×1.6-2.96 μm in size (Fig. 1). Colonies were circular, smooth, slightly convex and beige- to cream-coloured

on M2 medium.

Temperature range for growth was determined between 4 and 45□. The initial growth pH range (3-10) was tested by using 5 ml HLB medium supplemented with 200 µl M2 medium, adjusted with NaOH (5N) and HCl (5M) solution. HLB 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) on M2. The requirement for Na^+ was studied on M2 agar medium in the absence of Na^+, with the modification that distilled water was used instead of sea water and Na^+ was replaced by K^+. Each experiment was incubated at 25□ except temperature assay. Oxidase reaction was tested by using oxidase reagent (bioMérieux). Catalase activity was tested using a 3.0 % H_2O_2 solution. Hydrolysis of starch, Tween 20, 40 and 80 were determined as described previously by Cowan & Steel (1965). Other physiological and biochemical tests were performed with the API 20E and API 20NE systems (bioMérieux) and the inoculum was prepared by suspending cells in a 3% (w/v) NaCl solution. The API ZYM system (bioMérieux) was used to determine the activity of some enzymes. Production of bacteriochlorophyll α (Bchl α) was determined by spectrophotometric analysis as described by Martens *et al.* (2006). The utilization of various substrates for growth was detected by spectrophotometer at 600nm after incubation for 3 days at 25℃. The following single substrates were added to the MM medium (l^{-1}, 7.0 g $MgSO_4$·7H2O, 24 g NaCl, 0.35 g KCl, 1.0 g NH_4NO_3, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 0.08 g KBr, 2.6 mg NaF, 27 mg H_3BO_3, 31 mg $NaHCO_3$, 0.05 g $CaCl_2$, 2.8 mg $FeSO_4·7H_2O$, 0.1 mg $ZnSO_4·7H_2O$, 24 mg $SrCl_2·6H_2O$) as sole carbon sources to a final concentration of 1.0 g l^{-1}: D-glucose, D-fructose, D-galactose, sucrose, trehalose, L-rhamnose, D-sorbitol, cellobiose, acetate, citrate, succinate, β-hydroxy butyric acid,

D-glucosamine, L-glutamate, serine, leucine, methionine, betaine and glycerol.

Susceptibility to different antibiotics was tested on M2 agar plates by using discs (OXOID) diffusion method at the following compounds: ceftriaxone (30 μg), cephradine (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), erythromycin (15 μg), cefoperazone (75 μg), ciprofloxacin (5 μg), clindamycin (2 μg), doxycycline hydrochloride (30 μg), neomycin (10 μg), tetracycline (30 μg), cephalexin (30 μg), ampicillin (10 μg), furazolidone (15 μg), metronidazole (5 μg), cephazolin (30 μg), lincomycin (2 μg), minocycline (30 μg), norfloxacin (10 μg), kanamycin (30 μg), vancomycin (30 μg), trimethoprim (25 μg), piperacillin (100 μg), ofloxacin (5 μg), rifampicin (5 μg), carbenicillin (100 μg), polymyxin B (300 U), streptomycin (10 μg), oxacillin (1 μg) and penicillin G (10 U). The plates were incubated at 25 °C for 3 days

The cellular polar lipids were extracted according to the procedures described by Komagata & Suzuki (1987) and separated on silica gel plates (DC Kieselgel 60F; Merck) by using two-dimensional thin-layer chromatography (TLC) and identified on the basis of their reaction with appropriate detection reagents (Vaskovsky *et al.*, 1975; Ryu & MacCoss, 1979). Phospholipids were detected as blue spots on a white ground by spraying Dittmer-Lester reagent. The presence of amino-containing lipids was identified by spraying ninhydrin reagent. Cell biomass of strain NH52F^T for polar lipid analysis was obtained from cultures grown in M2 at 25 °C.

For cellular fatty acid analysis, strain NH52F^T was harvested from marine agar 2216 agar plates (MA; Difco) after cultivation for 3 days at 25 °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 were performed on a gas chromatography (GC 6850; Agilent), and peaks were

identified with MIDI software (version 6.0). The predominant cellular fatty acids of strain NH52F^T were the straight-chain unsaturated and saturated fatty acids and hydroxy fatty acids. The compositions of fatty acids were $C_{12:0}$ 3-OH (2.9%), $C_{16:0}$ (3.0%), 11-methyl $C_{18:1}\omega7c$ (3.0%), $C_{10:0}$ 3-OH (3.9%), $C_{16:0}$ 2-OH (5.4%), $C_{18:1}\omega7c$ (71.9%) and an unknown fatty acid of equivalent chain-length 11.799 (5.7%). A relatively large amount of $C_{18:1}\omega7c$ is a feature of majority of species within the *Alphaproteobacteria* (Martens *et al.*, 2006). This fatty acid profile was in good agreement with those of the type strains of the genus *Leisingera*, although there were differences in the proportions of some fatty acids. The fatty acid profiles of strain NH52F^T and recognized species of the related genera are given in Supplementary Table S1 available on IJSEM Online.

Cell mass of strain NH52F^T for DNA extraction was harvested from M2 plates after incubation at 25 °C for 3 days. Genomic DNA was extracted according to the method described by Ausubel *et al*. (1995). The 16S rRNA gene was PCR-amplified with two universal primers (Lane, 1991). The purification and sequencing of PCR product were performed as described by Liu & Shao (2005). A BLAST analysis with the nearly complete 16S rRNA gene sequence (1429 nt) of strain NH52F^T was performed to obtain the similar sequences. The determined sequences were manually aligned by using DNAMAN (Version 6; Lynnon Biosoft) and evolutionary distances were then computed according to the Kimura two-parameter model analysis (Kimura, 1980) also carried out by using the DNAMAN program. 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 (mol %) was determined to be 60.5% by reverse high-performance liquid chromatography (HPLC) according to the method of Tamaoka & Komagata (1984). This value was similar to that of the genus *Phaeobacter* (55.7-64.9 mol%), *Marinovum* (60 mol%) and

Leisingera (60.5±0.2 mol%), which is lower than the 10 mol% difference in DNA G+C content recommended for genus demarcation (Stackebrandt & Liesack, 1993), thus it is reasonable to place strain NH52F^T in the genus *Leisingera*.

Lists of phenotypic properties differentiating strain NH52F^T from closely related members within the *Roseobacter* clade were shown in Table 1. The major properties of NH52F^T are in accordance with those of emended description of the genus *Leisingera* (Schaefer *et al.*, 2002; Vandecandelaere *et al.*, 2008), including the inability to hydrolyse aesculin or gelatin, no indole production or acid production from glucose, failure to assimilate D-mannose and maltose, the presence of leucine arylamidase, susceptibility to erythromycin, and resistibility to vancomycin, trimethoprim and clindamycin. Accordingly, it is appropriate that NH52F^T be classified in the genus *Leisingera*.

Comparative 16S rRNA gene sequence analysis showed that strain NH52F^T was closely related to the members of the genera *Leisingera*, *Phaeobacter* and *Marinovum* within the family *Rhodobacteraceae*. Phylogenetic analysis indicated that strain NH52F^T and recognized *Leisingera* species formed a distinct cluster with a bootstrap resampling value of 51% (Fig. 2). Trees with similar topology were also generated using the maximum-likelihood and maximum-parsimony algorithms (data not shown). Strain NH52F^T exhibited the highest 16S rRNA gene sequence similarity to *L. methylohalidivorans* (96.7%) and *L. aquimarina* (95.8%), followed by *P. daeponensis* TF-218^T (96.0%), *P. inhibens* T5^T (95.9%), *P. gallaeciensis* BS107^T (95.7%), *P. arcticus* 20188^T (95.4%) and *M. algicola* ATCC 51440^T (94.1%). None of other type strains used in the phylogenetic analysis showed similarity higher than 94%. Genotypic (DNA G+C content) and phenotypic properties (Table 1) support the affiliation of strain NH52F^T as a member the

genera *Leisingera* including its colony colour, salinity range and a narrow substrate range. In addition, strain NH52F^T contained phosphatidylcholine (PC) (Table 1), which was distinguish it from the genera *Phaeobacter* and *Marinovum*. Moreover, fatty acids $C_{16:0}$ 2-OH and an unknown fatty acid (equivalent chain-length, 11.799) as minor components were present in strain NH52F^T and other *Leisingera* species, but absent in *Marinovum*. Thus, strain NH52F^T is more close to the member of genus *Leisingera* other than to those of *Phaeobacter* and *Marinovum*. On the other hand, strain NH52F^T distinguished itself from the recognized *Leisingera* species by the level of 16S rRNA sequence similarity (<97%, Stackebrandt & Goebel, 1994) and differences in phenotypic properties, including amylase, esterase lipase (C8) and acid phosphatase activity and antibiotic susceptibility (Table 1).

Based on the data presented, strain NH52F^T is considered to represent a novel species of the genus *Leisingera*, for which the name *Leisingera nanhaiensis* sp. nov. is proposed.

Description of *Leisingera nanhaiensis* sp. nov.

Leisingera nanhaiensis (nan.hai.en'sis. N. L. adj. referring to Nanhai, the South China Sea, the site where the type strain was isolated).

Cells are aerobic, Gram-staining-negative, motile rods, 0.62-0.8×1.6-2.96 μm in size. Colonies on M2 are circular, smooth, glistening, moist-appearing, slightly convex with entire margin, beige in colour and 1-1.5 mm in diameter after 5 days incubation at 25°C. Growth occurs at 4-37°C and pH 6.0-9.3, with optimal temperature at 25 °C and pH 7.0-8.5. NaCl is essential for growth. Growth occurs in the presence of 0.6-6.0 % (w/v) NaCl with optimal salinity from 1 to 4%. Bacteriochlorophyll α (Bchl α) is absent. Cells are positive for oxidase and catalase. Nitrate reduction, hydrolysis of starch, aesculin, Tween 20, 40 and 80, gelatin liquefaction and urease are negative. H_2S and indole are not produced. Voges-Proskauer reaction is negative. Arginine

dihydrolase, lysine decarboxylase, ornithine decarboxylase, β-galactosidase, β-glucosidase and tryptophan deaminase are negative. In assays with the API ZYM strips, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase are positive, but lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. Growth only occurs on complex substrates, such as yeast extract, tryptone and peptone from potatoes, beside betaine and methionine. No growth was detected on the following single carbon sources: D-glucose, D-fructose, D-galactose, sucrose, trehalose, L-rhamnose, D-sorbitol, cellobiose, acetate, citrate, succinate, D-glucosamine, L-glutamate, serine, leucine and glycerol except a faint growth was observed in the presence of β-hydroxy butyric. In addition, the type strain is susceptible to cefoperazone, ceftriaxone, erythromycin, ampicillin, kanamycin, norfloxacin, carbenicillin, polymyxin B, rifampicin, piperacillin, chloramphenicol and cephradine, but resistant to clindamycin, ciprofloxacin, lincomycin, furazolidone, cephazolin, penicillin G, doxycycline hydrochloride, cephalexin, tetracycline, metronidazole, minocycline, vancomycin, streptomycin, ofloxacin, trimethoprim, oxacillin, gentamicin and neomycin. The predominant fatty acids are (>1% of total fatty acids) $C_{18:1}\omega 7c$ (71.9%), followed by an unknown fatty acid (equivalent chain-length of 11.799; 5.7%), $C_{16:0}$ 2-OH (5.4%), $C_{10:0}$ 3-OH (3.9%), $C_{16:0}$ (3.0%), 11-methyl $C_{18:1}\omega 7c$ (3.0%) and $C_{12:0}$ 3-OH (2.9%).The major polar lipids are phosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid, an unidentified lipid and an aminolipid. The DNA G+C content is 60.5 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, NH52F^T (=LMG 24841^T=CCTCC AB 208316^T=MCCC 1A04178^T), was

isolated from a sandy sediment sample of the South China Sea at a depth of 157 m.

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Table 1. Phenotypic characteristics that differentiate strain NH52F^T from recognized species of the genera *Leisingera, Phaeobacter* and *Marinovum*

Taxa: 1, strain NH52F^T; 2, *L. methylohalidivorans* DSM 14336^T; 3, *L. aquimarina* LMG 24366^T; 4, *Phaeobacter*; 5, *M. algicola* DSM 10251^T. All strains are Gram-staining-negative, grow in the presence of Na^+ and are oxidase- and catalase-positive and unable to degrade Tween 80. For taxa 4, values are characteristics for all species in the genus. Data for reference strains from Vandecandelaere *et al.* (2008), Zhang *et al.* (2008), Yoon *et al.* (2007), Martens *et al.* (2006), Schaefer *et al.* (2002), Labrenz *et al.* (1999), Ruiz-Ponte *et al.* (1998), Lafay *et al.* (1995). +, positive; -, negative; W, weakly positive; ND, no data available.

Characteristic	**1**	**2**	**3**	**4**	**5**
Morphology	Rods/ovoid rods	Rods/ovoid rods	Ovoid	Ovoid rods	Rods or ovoid rods
Colony colour	Beige	No pigment	Dark beige-pink	Brown to dark brown or yellowish white	Beige/pinkish
Growth at 4□	+	+	+	-	-
Growth at 37□	W	-	+	+	+
Temperature (□)*	4-37 (25)	4-36 (27)	4-37 (20)	4-42 (19-37)	10-37 (25-30)
Salinity range (%)*	0.6-6 (1-4)	1-6.5 (2-4)	1-7	0.06-11.7 (1.2-4)	0.6-11.7
pH*	6-9.3 (7-8.5)	6.5-8.5 (7.7)	5.5-9(6.5-8)	5-10 (6-9)	6-9 (7.5)
Bchl α	-	-	ND	-†	-
Nitrate reduction	-	-	-	V	-
Acid from glucose	-	-	-	V	ND
Enzyme activities:					
Amylase	-	+	-	-	+
Gelatinase	-	-	W	-	+
Caseinase	-	-	-	-	ND
Esterase (C4)	W	-	-	V	ND
Acid phosphatase	+	-	-	V	ND
Naphthol-AS-BI-phosphohydrolase	+	W	W	-‡	ND

Utilization of:					
Betaine	+	+	+	V	ND
Methionine	+	+	-	+†	ND
L-Arabinose	-	-	-	V	+
D-Glucose	-	-	-	+	+
Maltose	-	-	-	V	+
D-Mannose	-	-	-	V	-
Citrate	-	-	-	V	+
Susceptibility to:					
Neomycin	-	W	ND	+†	-
Streptomycin	-	+	+	+†	-
Vancomycin	-	-	-	V	ND
Polar lipids#	PG,PE,PL,L,AL	PG,PE,PL,L,AL	ND	PC,PG,PE,L,AL,PL	PC,PG,PE,L,AL,PL
DNA G+C content (mol%)	60.5	60.5	61.4	55.7-64.9	60

*Ranges with optima shown in parentheses.

† No data available for the type strain of *Phaeobacter arcticus*

‡ No data available for the type strain of *Phaeobacter gallaeciensis.*

#PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid. AL, aminolipid; L, unidentified lipid;

Supplementary Table S1. Cellular fatty acid composition (%) of strain NH52F^T with recognized species of the genera *Leisingera, Phaeobacter* and *Marinovum*

Taxa: 1, strain NH52F^T; 2, *L. methylohalidivorans* DSM 14336^T; 3, *L. aquimarina* LMG 24366^T; 4, *M. algicola* DSM 10251^T; 5, *P. gallaeciensis* BS107^T; 6, *P. inhibens* T5^T; 7, *P. daeponensis* TF-218^T; 8, *P. arcticus* 20188^T. Values are percentage of total fatty acids. Data for reference strains from Yoon *et al.* (2007), Vandecandelaere *et al.* (2008), Zhang *et al.* (2008).

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Straight-chain fatty acids								
$C_{12:0}$		0.6			0.4	0.4	1.2	0.86
$C_{15:0}$								1.04
$C_{16:0}$	3.0	3.2	3.5	2.5	6.3	5.2	8.6	9.69
$C_{17:0}$		0.1		0.3	0.2	0.2	0.6	
$C_{18:0}$	0.6	0.5		2.5	1.3	2	2.4	0.53
Branch-chain fatty acids								
$C_{14:1}$ iso E			11.6					
Unsaturated fatty acids								
$C_{17:1}\omega 8c$	0.4	0.2		0.8				
$C_{18:1}\omega 7c$	71.9	77.5	71.6	81	74.5	70.8	57.7	44.63
Hydroxy fatty acids								
$C_{10:0}$ 3-OH	3.9	1.8	2.0	1.1	1.9	1.8	1.7	6.75
$C_{12:0}$ 3-OH	2.9	2.1	2.1	1.2	1.6	2	2.6	
$C_{16:0}$ 2-OH	5.4	4.7	4.2		2.7	2.8	5.6	3.95
$C_{18:1}$ 2-OH	0.7	0.6						0.59
11-Methyl $C_{18:1}\omega 7c$	3.0	4.7		9.6	7.8	11.8	16.6	18.1
Summed feature 3*	0.6	0.5		0.2	0.3	0.2	0.5	2.3
Unknown fatty acid (equivalent chain-length, 11.799)	5.7	3.1	2.7		2.6	2.7	2.3	10.88

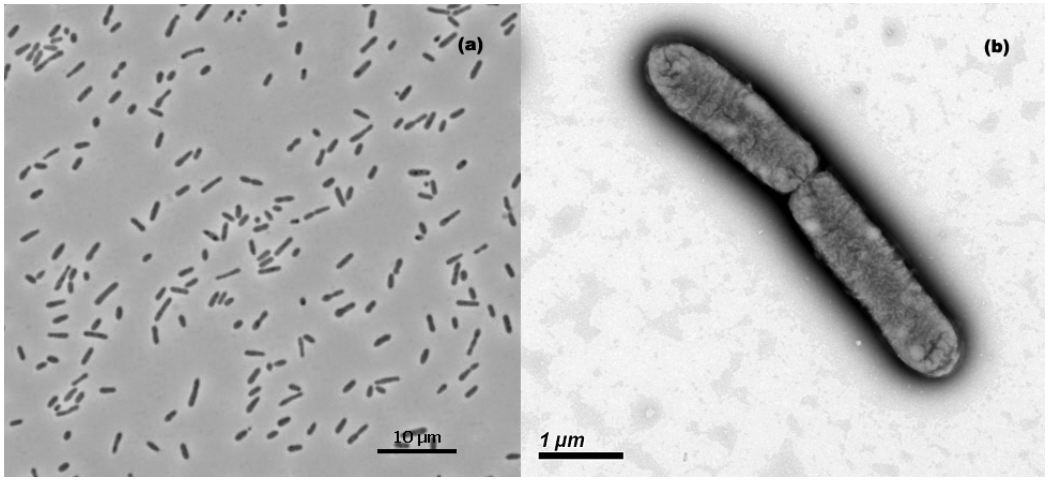
*summed feature 3 represented two or three fatty acids that could not be separated by the MIDI system: $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH

Legends to Figures:

Fig. 1. Cell morphology of strain NH52F^T grown on M2 agar plate for 24h at 25□: (a) phase contrast micrograph, Bar, 10 μm; (b), transmission electron micrographs of negatively stained cells, Bar, 1 μm.

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain NH52F^T and related taxa. Only bootstrap values above 50% (derived from 1000 replicates) are shown at the branching points. GenBank accession numbers of 16S rRNA gene sequences are shown in parentheses. Bar, 0.05 substitutions per nucleotide position.

288 Fig. 1

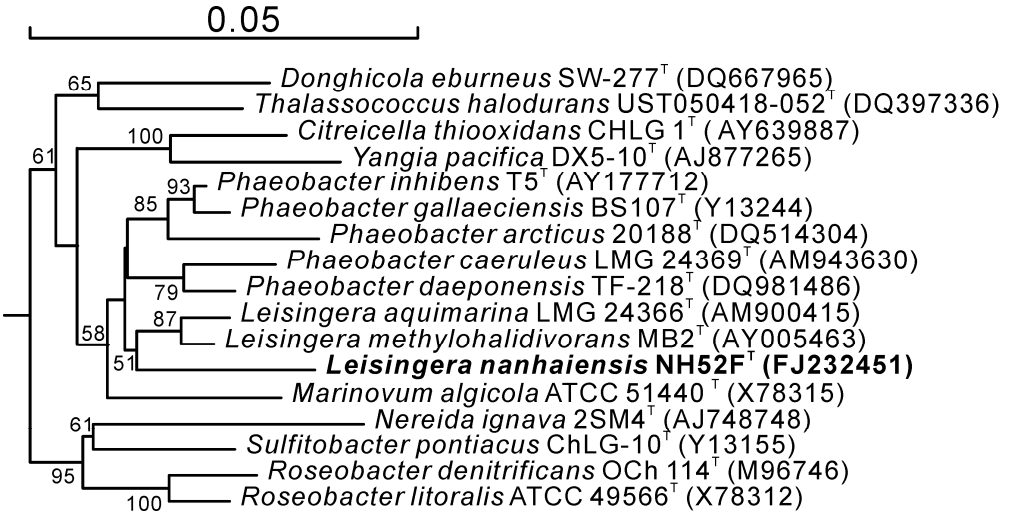


289

290 Fig. 1. Cell morphology of strain NH52F^{T} grown on M2 agar plate for 24h at 25□: (a) phase
291 contrast micrograph, Bar, 10 µm; (b), transmission electron micrographs of negatively stained
292 cells, Bar, 1 µm.

293

294 Fig. 2



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