Leisingera nanhaiensis sp. nov., isolated from marine sediment

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An aerobic, Gram-staining-negative, motile, rod-shaped bacterium, strain NH52F^T, was isolated from a sandy sediment sample taken from 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 sequences 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 the new isolate required the presence of 1–4 % (w/v) NaCl. The major fatty acids were C_{18:1}ω7c, C_{16:0} 2-OH, C_{10:0} 3-OH, C_{12:0} 3-OH, C_{16:0} and 11-methyl C_{18:1}ω7c. The DNA G + C content was 60.5 mol%. 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 16S rRNA gene similarity values demonstrated that the new isolate differed from recognized species of the genus *Leisingera*. 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) to accommodate Gram-stain-negative, aerobic, rod-shaped marine bacteria that are able to grow on methyl halides as a sole carbon source. Recently, emended descriptions of the genus *Leisingera* have been published by Martens *et al.* (2006) and Vandecastelaere *et al.* (2008) providing additional chemotaxonomic data and some further physiological and biochemical features. Currently, the genus includes two species of marine bacteria, *Leisingera methylohalidivorans* and *Leisingera aquimarina* (Schaefer *et al.*, 2002; Vandecastelaere *et al.*, 2008). The former species was isolated from a tide pool off the coast of California, USA. 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). In this study, we describe a new bacterial strain, NH52F^T, as the type strain of a novel

species of this genus on the basis of physiological and biochemical characteristics, 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 at a water depth of 157 m. In the laboratory, the sediment was suspended and diluted (1:100) using sterilized seawater, spread onto M2 agar plates and incubated at 25 °C for 1 week. The composition of M2 medium was (l⁻¹ seawater): 5 g sodium acetate, 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 ammonium nitrate, 0.2 g ammonium chloride 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 were 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

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

A table comparing the cellular fatty acid contents of strain NH52F^T with those of related species is available with the online version of this paper.

(Fig. 1). Colonies were circular, smooth, slightly convex and beige- to cream-coloured on M2 medium.

The temperature range for growth was determined between 4 and 45 °C. The initial growth pH range (3–10) was tested by using 5 ml HLB medium supplemented with 200 µl M2 medium, adjusted with NaOH (5 M) and HCl (5 M) 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 medium. 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 seawater and Na⁺ was replaced by K⁺. Each experiment was incubated at 25 °C, except for the temperature assay. The oxidase reaction was tested by using oxidase reagent (bioMérieux). Catalase activity was examined using a 3.0 % H₂O₂ solution. Hydrolysis of starch and Tweens 20, 40 and 80 was 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 *a* (Bchl *a*) was determined by spectrophotometric analysis as described by Martens *et al.* (2006). The utilization of various substrates for growth was detected with a spectrophotometer at 600 nm after incubation for 3 days at 25 °C. The following single substrates were added to MM medium 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-hydroxybutyric acid, D-glucosamine, L-glutamate, serine, leucine, methionine, betaine and glycerol. MM medium comprised (l⁻¹): 7.0 g MgSO₄·7H₂O, 24 g NaCl, 0.35 g KCl, 1.0 g NH₄NO₃, 1.0 g KH₂PO₄, 1.0 g K₂HPO₄, 0.08 g KBr, 2.6 mg NaF, 27 mg H₃BO₃, 31 mg NaHCO₃, 0.05 g CaCl₂, 2.8 mg FeSO₄·7H₂O, 0.1 mg ZnSO₄·7H₂O and 24 mg SrCl₂·6H₂O.

Susceptibility to different antibiotics was tested on M2 agar plates by using the disc diffusion method (Oxoid) with the

following compounds (µg per disc): ceftriaxone (30), cephadrine (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). 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 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 with Dittmer–Lester reagent. The presence of amino-containing lipids was revealed by spraying with ninhydrin reagent. The cell biomass for polar lipid analysis of strain NH52F^T 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 by GC (GC 6850; Agilent) and peaks were identified with MIDI software (version 6.0). The predominant cellular fatty acids of strain NH52F^T were straight-chain unsaturated and saturated fatty acids and hydroxy fatty acids. The main fatty acids were (>1 % of total fatty acids) C_{18:1}ω7c (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}ω7c (3.0 %) and C_{12:0} 3-OH (2.9 %). A relatively large amount of fatty acid C_{18:1}ω7c is a feature of the majority of species within the class *Alphaproteobacteria* (Martens *et al.*, 2006). This fatty acid profile was in good

Fig. 1. Cell morphology of strain NH52F^T grown on an M2 agar plate for 24 h at 25 °C. (a) Phase-contrast micrograph; bar, 10 µm. (b) Transmission electron micrograph of negatively stained cells; bar, 1 µm.

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 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 similar sequences. The sequence determined was aligned to sequences represented by the phylogeny shown on Fig. 2 by using DNAMAN (version 6; Lynnon Biosoft). Evolutionary distances were computed according to the Kimura two-parameter model (Kimura, 1980), also carried out with the DNAMAN software. 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 mol% by reverse HPLC according to the method of Tamaoka & Komagata (1984). This value was similar to that of members of the genera *Phaeobacter* (55.7–64.9 mol%), *Marinovum* (60 mol%) and *Leisingera* (60.5 ± 0.2 mol%). The difference in DNA G+C content between the new strain and the related genera was lower than the 10 mol% difference recommended for genus demarcation (Stackebrandt & Liesack, 1993) and thus it is reasonable to place strain NH52F^T in the genus *Leisingera*.

The phenotypic properties that differentiate strain NH52F^T from closely related members within the *Roseobacter* clade are shown in Table 1. The major properties of strain NH52F^T are in accordance with those given in the emended descriptions of the genus *Leisingera* (Schaefer *et al.*, 2002; Vandecastelaere *et al.*, 2008), including the inability to hydrolyse aesculin or gelatin, the lack of indole

or acid production from glucose, a failure to assimilate D-mannose or maltose, the presence of leucine arylamidase, susceptibility to erythromycin, and resistance to vancomycin, trimethoprim and clindamycin. Accordingly, it is appropriate that strain 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 the recognized species of the genus *Leisingera* 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* MB2^T (96.7%) and *L. aquimarina* LMG 24366^T (95.8%), followed by *Phaeobacter daeponensis* TF-218^T (96.0%), *Phaeobacter inhibens* T5^T (95.9%), *Phaeobacter gallaeciensis* BS107^T (95.7%), *Phaeobacter arcticus* 20188^T (95.4%) and *Marinovum algicola* ATCC 51440^T (94.1%). None of the other genera included in the phylogenetic analysis and represented by the type strains of the closest related species showed more than 94% 16S rRNA gene sequence similarity to the new strain. Genotypic (DNA G+C content) and phenotypic properties (Table 1) support the classification of strain NH52F^T as a member of the genus *Leisingera* including colony colour, salinity range and the narrow substrate range. In addition, strain NH52F^T contained phosphatidylcholine (PC) (Table 1), which distinguished it from members of the genera *Phaeobacter* and *Marinovum*. Moreover, fatty acids C_{16:0} 2-OH and an unknown fatty acid (equivalent chain-length, 11.799) were present in strain NH52F^T and other *Leisingera* species as minor components, but were absent from members of the genus *Marinovum*. Thus, strain NH52F^T is closer to the members of the genus *Leisingera* rather than to those of the genera *Phaeobacter* and *Marinovum*. Strain NH52F^T could be distinguished from the recognized species of the genus *Leisingera* by the level of 16S rRNA gene sequence

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 >50% (derived from 1000 replicates) are shown at the branching points. GenBank accession numbers are shown in parentheses. Bar, 0.05 substitutions per nucleotide position.

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⁺, are oxidase- and catalase-positive and are unable to degrade Tween 80. For taxon 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; v, variable.

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 °C	+	+	+	–	–
Growth at 37 °C	w	–	+	+	+
Temperature range (°C)	4–37	4–36	4–37	4–42	10–37
Optimum temperature (°C)	25	27	20	19–37	25–30
Salinity range (%; optimum)	0.6–6 (1–4)	1–6.5 (2–4)	1–7	0.06–11.7 (1.2–4)	0.6–11.7
pH range (optimum)	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 <i>a</i>	–	–	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-phospho-hydrolase	+	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

*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.

similarity (<97%, Stackebrandt & Goebel, 1994) and differences in phenotypic properties, including amylase, esterase lipase (C4) and acid phosphatase activities 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. fem. adj. *nanhaiensis* referring to Nanhai, the Chinese name for the South China Sea, from 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 margins, beige in colour and 1–1.5 mm in diameter after 5 days incubation at 25 °C. Growth occurs at 4–37 °C and at pH 6.0–9.3, with the optimal temperature being 25 °C and optimal pH being 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%. Bchl *a* is absent. Cells are positive for oxidase and catalase activities. Tests for nitrate reduction, hydrolysis of starch, aesculin and Tweens 20, 40 and 80 and liquefaction of gelatin and urease are negative. H₂S and indole are not produced. Voges–Proskauer reaction is negative. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, β -galactosidase, β -glucosidase and tryptophan deaminase activities are negative. In assays with API ZYM strips, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-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, as well as betaine and methionine. No growth is 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 faint growth was observed in the presence of β -hydroxybutyric acid. The type strain is susceptible to cefoperazone, ceftriaxone, erythromycin, ampicillin, kanamycin, norfloxacin, carbenicillin, polymyxin B, rifampicin, piperacillin, chloramphenicol and cephradine, but is resistant to clindamycin, ciprofloxacin, lincomycin, furazolidone, cephalosin, penicillin G, doxycycline hydrochloride, cephalosin, tetracycline, metronidazole, minocycline, vancomycin, streptomycin, ofloxacin, trimethoprim, oxacillin, gentamicin and neomycin. The predominant fatty acids are (>1 % of total fatty acids) C_{18:1} ω 7c, an unknown fatty acid (equivalent chain-length of 11.799), C_{16:0} 2-OH, C_{10:0} 3-OH, C_{16:0}, 11-methyl C_{18:1} ω 7c and C_{12:0} 3-OH. The major polar lipids are phosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid, an unidentified lipid and an aminolipid. 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 from the South China Sea from a depth of 157 m. The DNA G+C content of the type strain is 60.5 mol% (determined by HPLC).

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