Arenibacter nanhaiticus sp. nov., isolated from marine sediment of the South China Sea

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An aerobic, Gram-stain-negative, rod-shaped bacterial isolate, strain NH36A^T, was isolated from a sandy sediment sample from the South China Sea. Colonies of the isolate were dark orange on M2 agar. Optimal growth was observed at pH 7.0–8.5, 30 °C and in the presence of 0.5–4.0 % (w/v) NaCl. The major fatty acids were C_{15:0}, iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{15:1}, iso-C_{15:0} 3-OH, iso-C_{17:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1} ω7c). The DNA G + C content was 38.9 mol%. 16S rRNA gene sequence analysis revealed that strain NH36A^T was most closely related to members of the genus *Arenibacter*, exhibiting 94.3–96.2 % sequence similarity to the type strains of *Arenibacter* 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 *Arenibacter*. The name *Arenibacter nanhaiticus* sp. nov. is proposed and the type strain is NH36A^T (=LMG 24842^T=CCTCC AB 208315^T=MCCC 1A04137^T).

The South China Sea is a marginal sea in the south of China. It is a part of the Pacific Ocean, encompassing an area from Singapore to the Strait of Taiwan of around 3,500,000 km². It is one of the largest sea bodies after the five oceans and it has a remarkable amount of biological diversity. According to studies made by the Department of Environment and Natural Resources, The Philippines, this body of water holds one third of the world's marine biodiversity, thereby making it a very important ecosystem.

The genus *Arenibacter* belongs to the family *Flavobacteriaceae* within the phylum 'Bacteroidetes' and was proposed by Ivanova *et al.* (2001). Currently, it accommodates five species: *Arenibacter latericius*, *A. troitsensis*, *A. certesii*, *A. palladensis* and *A. echinorum* (Ivanova *et al.*, 2001; Nedashkovskaya *et al.*, 2003, 2004, 2006, 2007). The recent isolation of novel species of the genus *Arenibacter* led Nedashkovskaya *et al.* (2006) to emend the description of the genus *Arenibacter*. In this study, a dark-orange-pigmented marine bacterial strain, NH36A^T, which is considered to be an *Arenibacter*-like organism on the basis of 16S rRNA gene sequence comparison results, is described. Accordingly, the aim of the present work was to establish the exact taxonomic position of strain NH36A^T by using a polyphasic approach involving phenotypic, genetic and chemotaxonomic analyses.

Strain NH36A^T was isolated from a sandy sediment sample containing shell fragments collected from a water depth of 36 m in the South China Sea (19° 44.775' N 111° 6.192' E) in May 2007. The supernatant of sediment slurry was diluted and spread on M2 agar plates, which were then incubated at 25 °C for 1 week. M2 contained (per litre of 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 g NH₄NO₃, 0.2 g NH₄Cl and 15 g agar, adjusted to about pH 7.5–7.6. Strain NH36A^T was picked out and subsequently purified three times on M2 by streaking. The strain was obtained from the plate by gently scraping the surface of the medium and preserved as a 20 % (v/v) glycerol suspension at –80 °C. Cell morphology was examined by light microscopy (model 50i; Nikon) and transmission electron microscopy (model JEM-1230; JEOL) using cells from exponentially growing cultures.

Growth in the absence of NaCl was investigated in M2 medium lacking Na⁺. Growth at various NaCl concentrations (0.5–8.0 %, w/v, at 0.5 % intervals and 8–25 %, w/v, at 2 % intervals) was investigated in M2 medium. Growth at different temperatures (4, 10, 15, 20, 25, 30, 35, 40, 45 °C) was measured on M2 medium. The initial pH range for growth was determined in 5 ml HLB medium supplemented with 200 µl M2 medium, which was adjusted to various pH (2.5–10.0 at intervals of 0.5) using HCl (5 M) and NaOH (5 M). HLB was modified from Luria–Bertani medium (Sambrook *et al.*, 1989), with the concentration of NaCl increased to 30 g l^{–1}. Growth was measured spectrophotometrically at 600 nm after incubation for 2 days. The

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presence of flexirubin-type pigments was investigated as described by Christakis *et al.* (2005). The oxidase reaction was tested by using oxidase reagent (bioMérieux). Catalase activity was tested using a 3 % H₂O₂ solution. Hydrolysis of starch, Tween 20, Tween 40 and Tween 80 was determined as described by Cowan & Steel (1965). Degradation of alginate, cellulose and chitin was tested as described previously (Ivanova *et al.*, 2001). Other physiological and biochemical tests were performed with the API 20E and API 20NE systems (bioMérieux); the NaCl concentration was increased to 30 g l⁻¹ in API NaCl medium (bioMérieux). The API ZYM system (bioMérieux) was used to determine the activity of some enzymes. Oxidation of various substrates was determined by using the Biolog system as described previously (Ivanova *et al.*, 1998). GN2 microplates were selected and, after adding 150 µl liquid culture with the NaCl concentration increased to 2.4 %, plates were incubated at 30 °C.

Susceptibility to different antibiotics was tested on M2 agar plates by using discs (Oxoid) containing 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), cephalixin (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). Plates were incubated at 30 °C for 2 days.

Cellular polar lipids were extracted as described by Komagata & Suzuki (1987), separated on silica gel plates (DC Kieselgel 60F; Merck) by using TLC and identified according to their reactions with appropriate detection reagents. Cell biomass of strain NH36A^T for polar lipid analysis was obtained from cultures grown in M2 for 3 days at 30 °C. The polar lipid composition of the isolate is consistent with that given in the emended description of the genus *Arenibacter* (Nedashkovskaya *et al.*, 2006), consisting mainly of phosphatidylethanolamine.

For cellular fatty acid analysis, strain NH36A^T was harvested from marine agar 2216 (Difco) plates after cultivation for 3 days at 25 °C. 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 NH36A^T were straight-chain, branched-chain saturated and unsaturated fatty acids, namely C_{15:0} (9.4 %), iso-C_{15:0} (11.6 %), iso-C_{17:0} 3-OH (9.4 %), anteiso-C_{15:0}

Table 1. Cellular fatty acid compositions (%) of strain NH36A^T and *Arenibacter* type strains

Taxa: 1, strain NH36A^T (*A. nanhaiticus* sp. nov.); 2, *A. latericius* (data for four strains; results for the type strain in parentheses); 3, *A. troitsensis* KMM 3674^T; 4, *A. certesii* KMM 3941^T; 5, *A. palladensis* KMM 3961^T; 6, *A. echinorum* KMM 6032^T. Data for reference strains are from Nedashkovskaya *et al.* (2007).

Fatty acid	1	2	3	4	5	6
iso-C _{15:0}	11.6	6.9–15.8 (8.1)	6.8	7.7	8.7	6.4
anteiso-C _{15:0}	9.1	4.8–13.5 (9.3)	3.2	6.3	3.3	1.8
iso-C _{15:1}	14.2	4.9–14.0 (14.0)	12.2	7.2	12.7	13.1
anteiso-C _{15:1}	2.0	0.6–2.9 (2.9)	0.6	0.8	0.5	0.5
C _{15:0}	9.4	4.2–16.0 (14.2)	13.6	11.5	15	22
C _{15:1} ω6c	1.4	1.0–2.3 (2.3)	1.2	1.9	2.6	4.2
iso-C _{16:0}	0.5	0.5–1.3 (0.5)	0.3	1.7	0.2	1.0
C _{16:0}	2.3	1.1–2.7 (1.9)	1.5	1.0	0.6	0.6
iso-C _{17:1} ω9c	3.5	2.2–4.6 (2.9)	5.3	4.7	4.0	3.9
C _{17:1} ω8c	0.5	0.5–2.0 (1.3)	0.9	2.4	0.5	1.1
C _{17:1} ω6c	0.6	0.7–2.9 (2.4)	1.1	3.0	1.4	2.9
C _{15:0} 2-OH	0.5	0.6–1.0 (0.6)	0.4	0.6	0.4	0.6
iso-C _{15:0} 3-OH	7.2	4.6–5.7 (5.6)	5.1	3.5	5.3	3.5
C _{15:0} 3-OH	1.3	0–1.4 (0)	1.6	0.6	2.2	2.0
iso-C _{16:0} 3-OH	3.2	2.1–5.7 (2.1)	2.2	7.2	1.6	1.0
C _{16:0} 3-OH	3.1	0.6–1.3 (1.3)	2.2	0.8	2.0	1.0
iso-C _{17:0} 3-OH	9.4	6.9–14.4 (6.9)	21.9	13.3	17.4	10.5
C _{17:0} 2-OH	1.1	2.1–5.1 (2.1)	1.7	3.8	1.0	0.3
Summed feature 3*	10.3	9.8–11.9 (9.8)	9.6	13.5	11.1	10.7

*Summed features are groups of two or three fatty acids that cannot be separated by GC using the MIDI system. Summed feature 3 comprised iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

(9.1 %), iso-C_{15:0} 3-OH (7.2 %), iso-C_{15:1} (14.2 %) and summed feature 3 (10.3 %; comprising iso-C_{15:0} 2-OH and/or C_{16:1} ω7c). The presence of a significant amount of iso-C_{17:0} 3-OH (9.4 % in strain NH36A^T; 6.9–21.9 % in the type strains of the five *Arenibacter* species) supports placement of the isolate in the family *Flavobacteriaceae* (Nedashkovskaya *et al.*, 2006). This fatty acid profile was similar to those of the type strains of *Arenibacter* species, although there were differences in the proportions of some fatty acids (Table 1). These chemotaxonomic data show that strain NH36A^T appears to belong to the genus *Arenibacter*.

Genomic DNA was prepared from cells cultivated on M2 agar plates according to the extraction protocol described by Ausubel *et al.* (1995). To detect the precise taxonomic position of the isolate, the almost-full-length 16S rRNA gene sequence was determined by PCR amplification and subjected to sequencing. The determined sequences were aligned manually with reference 16S rRNA gene sequences available in GenBank and evolutionary distances were then computed by using the program DNAMAN with the Kimura two-parameter correction (Kimura, 1980); a phylogenetic tree was constructed (Fig. 1) according to the neighbour-joining method (Saitou & Nei, 1987). To evaluate the phylogenetic tree, bootstrap analysis with 1000 sample replications was performed.

The DNA G + C content of strain NH36A^T was 38.9 mol%, determined by reversed-phase HPLC according to the method of Tamaoka & Komagata (1984), which is in the range for members of the genus *Arenibacter* (Table 2).

Morphological, physiological and biochemical characteristics of strain NH36A^T are given in the species description and Table 2. Cells were rod-shaped and motile by means of gliding (Fig. 2). The isolate grew well on M2 medium. Colonies were circular, moist-appearing and convex with an entire margin and a smooth surface. Cell growth occurred at salinities of 0.5–6.5 % (w/v). Optimal growth occurred in the presence of 0.5–4.0 % (w/v) NaCl. No growth occurred in the absence of NaCl or above 8 % (w/v) NaCl. Within the tested temperature range (4–45 °C), growth occurred at 10 °C, but not at 45 °C, with optimum growth at 30 °C. Optimal growth occurred between pH 7.0 and 8.5. Weak growth occurred at pH 6.1 and 9.6, but there was no growth at pH 5.5 or 10.0. Strain NH36A^T could be distinguished from other *Arenibacter* species by its inability to utilize L-arabinose, the absence of β-glucosidase and susceptibility to penicillin G. In addition, the inability to generate acid from D-glucose or L-rhamnose, susceptibility to ampicillin and the requirement of Na⁺ ions for growth distinguished this strain from *A. palladensis* and *A. echinorum*; motility by gliding, the failure to reduce nitrate, and the absence of α-chymotrypsin and urease activities

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain NH36A^T and strains of related species belonging to the family *Flavobacteriaceae*. Only bootstrap values above 50 % are shown (1000 resamplings) at branch points. Bar, 0.05 substitutions per nucleotide position.

Table 2. Phenotypic characteristics that distinguish *Arenibacter nanhaiticus* NH36A^T from other *Arenibacter* species

Taxa: 1, strain NH36A^T (*A. nanhaiticus* sp. nov.); 2, *A. latericius* KMM 426^T; 3, *A. troitsensis* KMM 3674^T; 4, *A. certesii* KMM 3941^T; 5, *A. palladensis* KMM 3961^T; 6, *A. echinorum* KMM 6032^T. Data are from Ivanova *et al.* (2001), Nedashkovskaya *et al.* (2003, 2004, 2006, 2007) and this study. All strains are positive for the following: respiratory-type metabolism; oxidase, catalase, acid and alkaline phosphatases, α - and β -galactosidases, α -glucosidase, *N*-acetyl- β -glucosaminidase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α -mannosidase and α -fucosidase; utilization of D-glucose, D-lactose, trehalose, raffinose and D-mannose; and susceptibility to lincomycin and resistance to kanamycin, gentamicin, neomycin and polymyxin B. All strains are negative for the following: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase; production of flexirubin-type pigments and indole; degradation of agar, alginate, cellulose, chitin, starch and Tween 80; acid production from inositol and mannitol; and utilization of inositol. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
Gliding motility	+	–	–	–	+	+
Cell diameter (μ m)	0.45–0.8	0.4–0.6	0.4–0.7	0.4–0.7	0.4–0.5	0.4–0.5
Cell length (μ m)	1.4–3.8	2.1–5.0	3–5	3–5	1.6–2.3	1.5–2.7
Na ⁺ requirement	+	+	+	+	–	–
Nitrate reduction	–	+	+	+	–	–
H ₂ S production	–	–	+	–	–	–
Voges–Proskauer reaction	+	–	–	–	–	+
Degradation of:						
Gelatin	–	–	+	–	–	–
Urea	–	+	–	+	–	–
Tween 20	–	–	–	–	–	+
Tween 40	–	–	+	–	–	+
Tolerance to:*						
Temperature (°C)	10–40 (30)	10–42 (28)	10–42 (30)	4–38 (38)	4–38 (23–25)	4–35 (24–26)
pH	6.1–9.6 (7.0–8.5)	6.0–10.0 (7.5–8.5)	5.5–10.0 (7.5–8.5)	ND	ND	ND
Salt concentration (%)	0.5–6.5 (0.5–4.0)	1–8	1–6	1–10	0–10	0–8
Utilization of:						
D-Galactose	+	+	ND	+	+	–
Maltose	+	+	–	+	+	+
L-Arabinose	–	+	+	+	+	+
Glycerol	–	+	–	–	–	–
<i>N</i> -Acetyl- β -glucosamine	+	+	–	+	+	–
Mannitol	–	–	–	–	–	+
Malate	–	–	–	–	–	+
Citrate	–	–	–	–	–	+
Acid production from:						
D-Glucose	–	+	–	+	+	+
Melibiose	–	+	–	+	–	+
L-Rhamnose	–	+	–	–	+	+
Production of:						
α -Chymotrypsin	–	+	+	+	–	–
β -Glucuronidase	–	–	+	–	–	+
β -Glucosidase	–	+	+	+	+	+
Susceptibility to:						
Ampicillin	+	+	–	+	–	–
Carbenicillin	+	+	–	–	–	–
Tetracycline	–	–	+	–	–	+
Penicillin G	+	–	–	–	–	–
Streptomycin	–	–	–	–	ND	+
DNA G + C content (mol%)	38.9	37–38	40	37.7	40	39–40

*Ranges are given; optimal conditions are indicated in parentheses.

Fig. 2. Microscopic images of strain NH36A^T (*A. nanhaiticus* sp. nov.). (a–c) Transmission electron micrographs of negatively stained cells grown on M2 agar medium for 24 h at 30 °C; bars, 1 µm. (d) Photomicrograph of vegetative cells viewed by phase-contrast microscopy; bar, 10 µm.

separated the isolate from *A. latericius*, *A. troitsensis* and *A. certesii*.

The 16S rRNA gene sequence of strain NH36A^T comprising 1464 nt was determined after PCR amplification. The isolate showed highest 16S rRNA gene sequence similarities with the type strains of members of the genus *Arenibacter*: 96.2 % with *A. palladensis*, 96.0 % with *A. echinorum*, 95.8 % with *A. troitsensis*, and 94.3 % with *A. certesii* and *A. latericius*. Other members of the family *Flavobacteriaceae* were even more distantly related to strain NH36A^T, with 16S rRNA gene sequence similarities of ≤92.1 % (92.1 % similarity with *Maribacter sedimenticola* KMM 3903^T). Phylogenetic analysis of 16S rRNA gene sequences from organisms with validly published names revealed that strain NH36A^T and the five recognized species of *Arenibacter* belonged to the same clade. Within the clade, strain NH36A^T, *A. echinorum*, *A. troitsensis* and *A. palladensis* formed a separate subclade, which clustered robustly (100 % bootstrap support, 1000 replicates) with the branch formed by *A. latericius* and *A. certesii* (Fig. 1). The same topology was also found in trees based on the maximum-likelihood and maximum-parsimony algorithms (data not shown). The results of phylogenetic analysis and the relative low 16S rRNA gene sequence similarities to *Arenibacter* species (94.3–96.2 %) suggest that strain NH36A^T represents a novel species within the genus *Arenibacter*.

Although strain NH36A^T formed a coherent cluster with *A. echinorum*, *A. palladensis* and *A. troitsensis* at a low bootstrap resampling value of 65 %, identical morphological, physiological and chemotaxonomic characteristics (Tables 1 and 2) strongly support placing strain NH36A^T in the genus *Arenibacter*. Hence, it is proposed that strain NH36A^T represents a novel species in the genus *Arenibacter*, *Arenibacter nanhaiticus* sp. nov.

Description of *Arenibacter nanhaiticus* sp. nov.

Arenibacter nanhaiticus (nan.hai'ti.cus. N.L. masc. adj. *nanhaiticus* pertaining to Nanhai, the Chinese name for the South China Sea, the site where the type strain was isolated).

Cells are aerobic, Gram-stain-negative rods (0.45–0.8 µm wide and 1.4–3.8 µm long) that are motile by means of gliding. Colonies on M2 are circular, moist-appearing, glistening with a smooth surface, low convex with entire margins, dark orange in colour and 1–3 mm in diameter after 5 days incubation at 30 °C. Growth occurs at 10–40 °C and pH 6.1–9.6. The optimal temperature for growth is 30 °C. Optimal pH for growth is 7.0–8.5. NaCl is essential for growth. Growth is detected in the presence of 0.5–6.5 % (w/v) NaCl. The optimal NaCl concentration for growth is 0.5–4.0 %. Flexirubin-type pigments are not produced. Positive for oxidase and catalase. Negative for nitrate reduction, hydrolysis of starch, gelatin liquefaction and urease activity. H₂S and indole are not produced. In assays with the API 20E system, β-galactosidase is positive. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase. In API ZYM strips, alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are positive. Trypsin, α-galactosidase and α-glucosidase are weakly positive. Esterase (C4), esterase lipase (C8), lipase (C14), α-chymotrypsin, β-glucuronidase and β-glucosidase are negative. The Voges–Proskauer test is positive. In Biolog GN2 microplates, positive for oxidation of dextrin, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose,

D-mannose, methyl β -D-glucoside, raffinose, D-sorbitol, sucrose, trehalose, turanose and acetic acid. L-Glutamic acid is oxidized weakly. Susceptible to cefoperazone, clindamycin, ciprofloxacin, ceftriaxone, erythromycin, lincomycin, furazolidone, ampicillin, cephalosporin, carbenicillin, rifampicin, piperacillin, penicillin G, chloramphenicol and doxycycline hydrochloride, but resistant to cephalosporin, kanamycin, norfloxacin, tetracycline, metronidazole, minocycline, vancomycin, streptomycin, polymyxin B, ofloxacin, trimethoprim, oxacillin, cephradine, gentamicin and neomycin. Predominant fatty acids are straight-chain unsaturated and saturated, and branched-chain unsaturated and saturated fatty acids, namely iso-C_{15:1}, iso-C_{15:0}, C_{15:0}, iso-C_{17:0} 3-OH, anteiso-C_{15:0}, iso-C_{15:0} 3-OH, iso-C_{17:1} ω 9c, iso-C_{16:0} 3-OH, C_{16:0} 3-OH, C_{16:0} and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c). The major polar lipid is phosphatidylethanolamine. Other phenotypic characteristics are given in Table 2.

The type strain is NH36A^T (=LMG 24842^T=CCTCC AB 208315^T=MCCC 1A04137^T), isolated from a sandy sediment sample from the South China Sea. The DNA G+C content of the type strain is 38.9 mol% (determined by HPLC).

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