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Arenibacter nanhaiticus sp. nov., a novel marine bacterium isolated from marine sediment of South China Sea

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The GenBank accession number for the 16S rRNA gene sequence of *Arenibacter nanhaiticus* NH36A^T is EU999955.

Summary: An aerobic, Gram-staining-negative, rod-shaped bacterial isolate, strain NH36A^T, was isolated from a sandy sediment sample in 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% (w/v) NaCl. The major fatty acids were namely $C_{15:0}$, 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 $C_{16:1}\omega7c$). The DNA G+C content was 38.9%. The 16S rRNA gene sequence analysis revealed that strain NH36A^T was related most closely to members of the genus *Arenibacter*, exhibiting 94.3-96.2 % sequence similarity to the type strains of *Arenibacter*. 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. 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. It has a remarkable amount of biological diversity. According to studies made by the Department of Environment and Natural Resources, Philippines, this body of water holds one third of the all world's marine biodiversity, thereby making it a very important area for the ecosystem.

The genus *Arenibacter* belongs to the family *Flavobacteriaceae* within the *Cytophaga-Flavobacterium-Bacteroides* phylum and proposed by Ivanova *et al.* (2001) currently accommodate five species, including *Arenibacter latericius*, *Arenibacter troitsensis*, *Arenibacter certesii*, *Arenibacter palladensis* and *Arenibacter echinorum* (Ivanova *et al.*, 2001;

Nedashkovskaya *et al.*, 2003, 2004, 2006, 2007). Recently, some novel species of the genus *Arenibacter* have been found and lead Nedashkovskaya *et al.* (2006) to emend the description of the genus *Arenibacter*. In this study, we describe a dark-orange-pigmented marine bacterial strain NH36A^T, which is considered to be an *Arenibacter*-like organism on the basis of the results of 16S rRNA sequence comparison. Accordingly, the aim of the present work is 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 (111°6.192′E, 19°44.775′N) in May 2007. The supernatant of sediment slurry was diluted and spread on M2 agar plates, which was then incubated at 25 °C for 1 week. M2 contained (per liter of 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 g NH_4NO_3, 0.2 g NH_4Cl and 15 g agar, adjusted to 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 . 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 %, w/v, at 0.5% intervals and 8-25%, at 2% intervals) was investigated in M2 medium. Growth at different temperatures (4, 10, 15, 20, 25, 30, 35, 40, 45) was measured on M2. The initial pH range for growth was determined in 5ml HLB medium supplemented with

63 200 µl M2 medium, which was adjusted to various pH values (2.5-10.0 at intervals of 0.5 pH units)
64 using HCl (5N) and NaOH (5N). HLB was modified from Luria-Bertani (LB) medium (Sambrook
65 *et al*., 1989), with the concentration of NaCl increased to 30 g l^{-1}. The growth was measured by
66 using the spectrophotometer at 600 nm after incubation for 2 days. The presence of flexirubin-type
67 pigments was investigated as described by Christakis *et al*. (2005). Oxidase reaction was tested by
68 using oxidase reagent (bioMe´rieux). Catalase activity was tested using a 3% H_2O_2 solution.
69 Hydrolysis of starch, Tween 20, Tween 40 and Tween 80 were determined as described by Cowan
70 & Steel (1965). Degradation of alginate, cellulose and chitin were tested as described previously
71 (Ivanova *et al.*, 2001). Other physiological and biochemical tests were performed with the API 20E
72 and API 20NE systems (bioMe´rieux) and the concentration of NaCl was increased to 30 sg l^{-1} in
73 API NaCl medium (bioMe´rieux). The API ZYM system (bioMe´rieux) was used to determine the
74 activity of some enzymes. The oxidation of various substrates was determined by using the
75 BIOLOG system as described previously (Ivanova *et al*., 1998). GN2 Microplate was selected
76 and after adding 150 µl liquid culture with the NaCl concentration increased to 2.4%, the plate was
77 incubated at 30 °C.
78 Susceptibility to different antibiotics was tested on M2 agar plates by using discs (OXOID)
79 containing the following compounds: ceftriaxone (30 µg), cephradine (30 µg), chloramphenicol (30
80 µg), gentamicin (10 µg), erythromycin (15 µg), cefoperazone (75 µg), ciprofloxacin (5 µg),
81 clindamycin (2 µg), doxycycline hydrochloride (30 µg), neomycin (10 µg), tetracycline (30 µg),
82 cephalexin (30 µg), ampicillin (10 µg), furazolidone (15 µg), metronidazole (5 µg), cephazolin (30
83 µg), lincomycin (2 µg), minocycline (30 µg), norfloxacin (10 µg), kanamycin (30 µg), vancomycin
84 (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 30 °C for 2 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 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 in agreement with the emended description of the genus *Arenibacter* (Nedashkovskaya *et al.*, 2006); which mainly consist of phosphatidylethanolamine.

For cellular fatty acid analysis, strain NH36A^T was harvested from marine agar 2216 (MA; Difco) plates after cultivation for 3 days at 25 °C. The fatty acids were extracted according to the standard protocol of the Microbial Identification Ststem (MIDI, Sherlock). Analysis of the fatty acid methyl esters were performed on a GC (6850, Agilent), and peaks were identified with MIDI software (version 6.0). The predominant cellular fatty acids of strain NH36A^T were the 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-$C_{15:0}$ (9.1%), iso-$C_{15:0}$ 3-OH (7.2%), iso-$C_{15:1}$ (14.2%) and summed feature 3 (10.3%; comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). The presence of a significant amount of iso-$C_{17:0}$ 3-OH (6.9-21.9%) support it placed in the family *Flavobacteriaceae* (Nedashkovskaya *et al*., 2006). This fatty acid profile was similar to those of the type strains of *Arenibater* species, though there were differences in the proportions of some fatty acids (Table 2). These data of chemotaxonomic analyses support that strain NH36A^T appeared 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 strain studied, the almost-full length 16S rRNA gene sequence was determined by PCR amplification and subjected to sequencing. The determined sequences were manually aligned together with the reference 16S rRNA gene sequences available in the GeneBank database and evolutionary distances were then computed by using the DNAMAN program with the Kimura two-parameter correction (Kimura, 1980); a phylogenetic tree was constructed 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 (mol %) determined was 38.9% by reverse HPLC according to the method of Tamaoka & Komagata (1984), which is in the range of the genus *Arenibacter* (Table 1).

Morphological, physiological and biochemical characteristics of strain NH36A^T were given in the species description below or were shown in Table 1. Cells are rods and motile by means of gliding (Fig. 2). It grew well on M2 medium. Colonies were circular, moist-appearing, convex, and having an entire margin and a smooth surface. Cell growth occurred at salinity range of 0.5-6.5% (w/v). Optimal growth occurred at in the presence of 0.5-4% (w/v) NaCl. No growth occurred without addition 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. pH optima was between pH 7.0 and 8.5. A weak growth occurred at pH 6.1 and 9.6, but not at pH5.5 and 10.0. Strain NH36A^T could be distinguished from other *Arenibacter* species by the inability to utilize L-arabinose, absence of β-glucosidases and susceptibility to penicillin G. In addition, the inability to generate acid from D-glucose or L-rhamnose, susceptibility to ampicillin and requirement of Na^+ ions for growth distinguished this strain from *A. palladensis* and *A. echinorum*; while motility by

129 gliding, failure to reduce nitrate, and the absence of α-Chymotrypsin and urease activities separated
130 the new isolate from *A. latericius*, *A. troitsensis* and *A. certesii*.

131 16S rRNA gene sequence of strain NH36A^T comprising 1464nt was determined after PCR
132 amplification. The isolate showed the highest 16S rRNA gene sequence similarity with members of
133 the genus *Arenibacter,* 96.2% for *A. palladensis*, 96.0% for *A. echinorum*, 95.8% for *A. troitsensis,*
134 94.3% for *A. certesi* and *A. latericius*. The other members of the family *Flavobacteriaceae* were
135 even more distantly related to strain NH36A^T, with less than 92.1% similarity, such as *Maribacter*
136 *sedimenticola* (92.1%). Phylogenetic analysis of 16S rRNA gene sequences from organisms with
137 validly published names revealed that strain NH36A^T and the five recognized species of
138 *Arenibacter* belonged to the same clade. Within the clade, NH36A^T and *A. echinorum*, *A. troitsensis*
139 and *A. palladensis* formed a separate sub-clade, which clustered robustly (100% bootstrap support,
140 1000 replicates) with the branch formed by *A. latericius* and *A. certesii* (Fig. 1). The same topology
141 of tree was also found on basis of maximum-likelihood and maximum-parsimony algorithms (data
142 not shown). The results of phylogenetic analysis and the relative low sequences similarity of 16S
143 rRNA to *Arenibacter* speices (94.3~96.2%) suggested that strain NH36A^T represented a novel
144 species within the genus *Arenibacter*.

145 Although strain NH36A^T formed a coherent cluster with *A. echinorum*, *A.palladensis* and *A.*
146 *troitsensis* at a low bootstrap resampling value of 65%, identical morphological, physiological and
147 chemotaxonomic characteristics (Table 1, 2) strongly supported placing strain NH36A^T into the
148 genus *Arenibacter*. Hence, it is proposed that strain NH36A^T should be identified as a
149 representative of a novel species in the genus *Arenibcter* and termed *Arenibacter nanhaiticus* sp.
150 nov.

Description of *Arenibacter nanhaiticus* sp. nov

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

Cells are aerobic, Gram-staining-negative rods, 0.45-0.8 μm wide and 1.4-3.8 μm long and motile by means of gliding. Colonies on M2 are circular, moist-appearing, glistening, smooth surface, low convex with entire margin, dark orange in colour, 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 concentration of NaCl for growth is 0.5-4.0%. Flexirubin-type pigments are not produced. Cells are positive for oxidase and catalase. Nitrate reduction, hydrolysis of starch, gelatin liquefaction and urease are negative. H_2S and indole are not produced. In assays with the API 20E system, β-galactosidase is positive. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are negative. In API ZYM strips, alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphtol-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. Test for VP are positive. In Biolog GN2 Microplates, the following substrates are oxidized: dextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, D-cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannose, β-methyl-D-glucoside, D-raffinose, D-sorbitol, sucrose, D-trehalose,

turanose and acetic acid. L-Glutamic acid is weakly oxidized. In addition, the type strain is susceptible to cefoperazone, clindamycin, ciprofloxacin, ceftriaxone, erythromycin, lincomycin, furazolidone, ampicillin, cephazolin, carbenicillin, rifampicin, piperacillin, penicillin G, chloramphenicol and doxycycline hydrochloride, but resistant to cephalexin, kanamycin, norfloxacin, tetracycline, metronidazole, minocycline, vancomycin, streptomycin, polymyxin B, ofloxacin, trimethoprim, oxacillin, cephradine, gentamicin and neomycin. The predominant fatty acids of strain NH36A^T are the straight-chain unsaturated and saturated, and branched-chain unsaturated and saturated fatty acids (>2.0 % of total fatty acids), namely iso-$C_{15:1}$ (14.2%), iso-$C_{15:0}$ (11.6%), $C_{15:0}$ (9.4%), iso-$C_{17:0}$ 3-OH (9.4%), anteiso-$C_{15:0}$ (9.1%), iso-$C_{15:0}$ 3-OH (7.2%), iso-$C_{17:1}\omega 9c$ (3.5%), iso-$C_{16:0}$ 3-OH (3.2%), $C_{16:0}$ 3-OH (3.1%), $C_{16:0}$ (2.3%) and summed feature 3 (10.3%; comprising $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$). The major polar lipid is phosphatidylethanolamine. The DNA G+C content is 38.9 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, NH36A^T (=LMG 24842^T=CCTCC AB 208315^T=MCCC 1A04137^T), was isolated from a sandy sediment sample in the South China Sea.

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Table 1. Phenotypic characteristics that distinguish *Arenibacter nanhaiticus* NH36A^T from other *Arenibacter* species

Taxa: 1, *A. nanhaiticus* NH36A^T; 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; All of the strains were positive for the following characteristics: respiratory-type metabolism; oxidase, catalase, acid and alkaline phosphatases, α- and β-galactosidases, α- glucosidases, N-acetyl-β-glucosaminidase, leucine-, valine- and cystine-arylamidases, trypsin, naphthol-AS-BI-phosphohydrolase, α-mannosidase and α-fucosidase activities; utilization of D-glucose, D-lactose, trehalose, D-raffinose and D-mannose; susceptibility to lincomycin and resistance to kanamycin, gentamicin, neomycin and polymyxin B. All of the strains were negative for the following characteristics: arginine dihydrolase, lysine- and ornithine-decarboxylases and tryptophan deaminase activities; production of flexirubin-type pigments and indole; degradation of agar, alginate, cellulose, chitin, starch and Tween 80; acid production from inositol, mannitol; utilization of inositol. +, Positive; -, negative; ND, not determined; Data from Ivanova *et al.* (2001), Nedashkovskaya *et al.* (2003, 2004, 2006, 2007) and this study.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Gliding motility	+	-	-	-	+	+
Cell diameter (μm)	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 (μm)	1.4-3.8	2.1-5	3-5	3-5	1.6-2.3	1.5-2.7
Na^+ requirement	+	+	+	+	-	-
Nitrate reduction	-	+	+	+	-	-
H_2S production	-	-	+	-	-	-
VP reaction	+	-	-	-	-	+
Degradation of:						
Gelatin	-	-	+	-	-	-
Urea	-	+	-	+	-	-
Tween 20	-	-	-	-	-	+
Tween 40	-	-	+	-	-	+
Tolerancel of:*						
Temperature ( )	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)	6-10 (7.5-8.5)	5.5-10 (7.5-8.5)	ND	ND	ND
Salt concentratin (%)	0.5-6.5 (0.5-4)	1-8	1-6	1-10	0-10	0-8
Utilization of compounds:						
D-Galactose	+	+	ND	+	+	-
Maltose	+	+	-	+	+	+
L-Arabinose	-	+	+	+	+	+
Glycerol	-	+	-	-	-	-
N-Acetyl-β-glucosamine	+	+	-	+	+	-
Mannitol	-	-	-	-	-	+
Malate	-	-	-	-	-	+
Citrate	-	-	-	-	-	+
Acid production from:						

D-Glucose	-	+	-	+	+	+
D-Melibiose	-	+	-	+	-	+
L-Rhamnose	-	+	-	-	+	+
Production of:						
α-Chymotrypsin	-	+	+	+	-	-
β-Glucuronidase	-	-	+	-	-	+
β-glucosidases	-	+	+	+	+	+
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 with optima are indicated in parentheses.

Table 2. Cellular fatty acid compositon (%) of strain NH36A^T and *Arenibacter* type strains

Taxa: 1, *A. nanhaiticus* NH36A^T; 2, *A. latericius* (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 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
$C_{15:1}\omega 6c$	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
$C_{16:0}$	2.3	1.1-2.7 (1.9)	1.5	1	0.6	0.6
iso-$C_{17:1}\omega 9c$	3.5	2.2-4.6 (2.9)	5.3	4.7	4	3.9
$C_{17:1}\omega 8c$	0.5	0.5-2.0 (1.3)	0.9	2.4	0.5	1.1
$C_{17:1}\omega 6c$	0.6	0.7-2.9 (2.4)	1.1	3	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
iso-$C_{16:0}$ 3-OH	3.2	2.1-5.7 (2.1)	2.2	7.2	1.6	1
$C_{16:0}$ 3-OH	3.1	0.6-1.3 (1.3)	2.2	0.8	2	1
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	0.3
Summed feature 3*	10.3	9.8-11.9 (9.8)	9.6	13.5	11.1	10.7

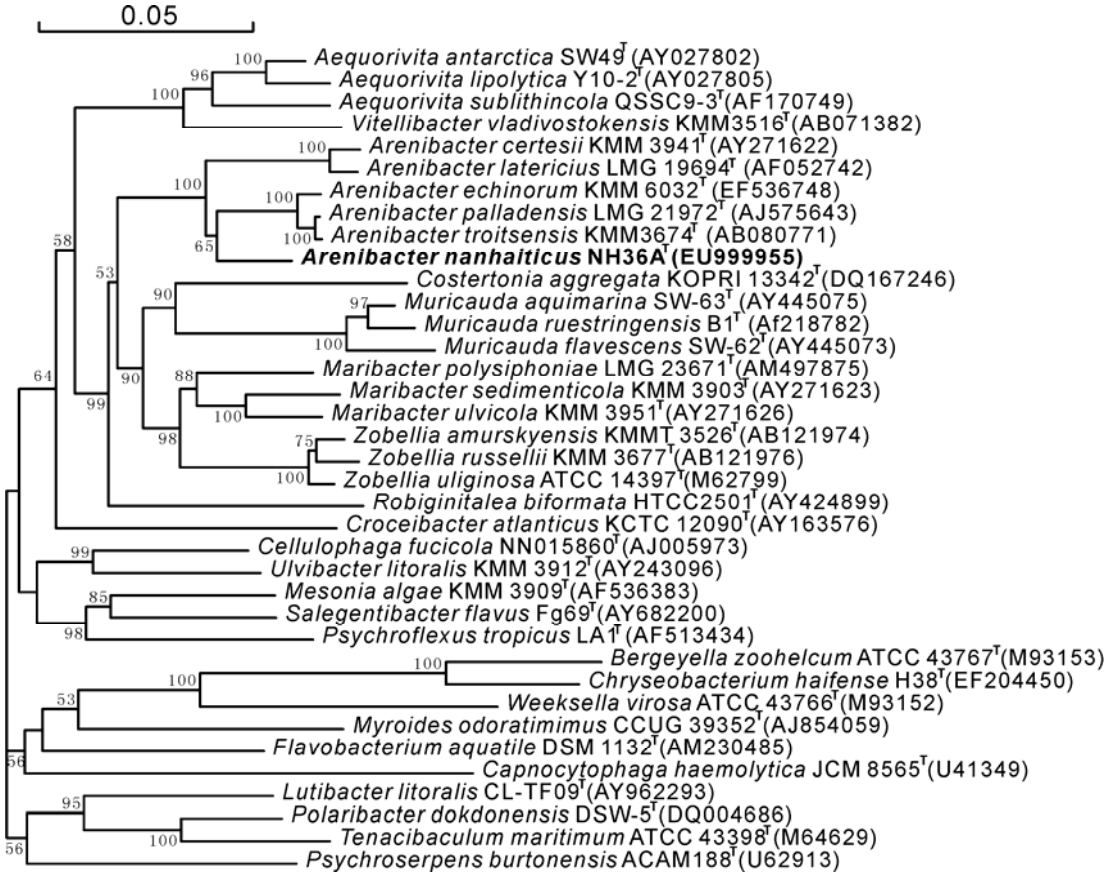
*Summed features are groups of two or three fatty acids that could not be separated by GC using the MIDI system. Summed feature 3 comprised iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega 7c$.

Legends to Figures:

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain NH36AT and other related species belonging to the family *Flavobacteriaceae*. Only bootstrap values above 50% are shown (1000 resamplings) at the branching points. Bar, 0.05 substitutions per nucleotide position.

Fig. 2. Microscopic images of *Arenibacter nanhaiticus* sp. nov. NH36A^{T} and bars were shown in images. (a-c), Transmission electron micrographs of negatively stained cells grown on M2 agar medium for 24h at 30 . (d), Photomicrograph of vegetative cells viewed by phase-contrast microscopy.

272 **Fig. 1**

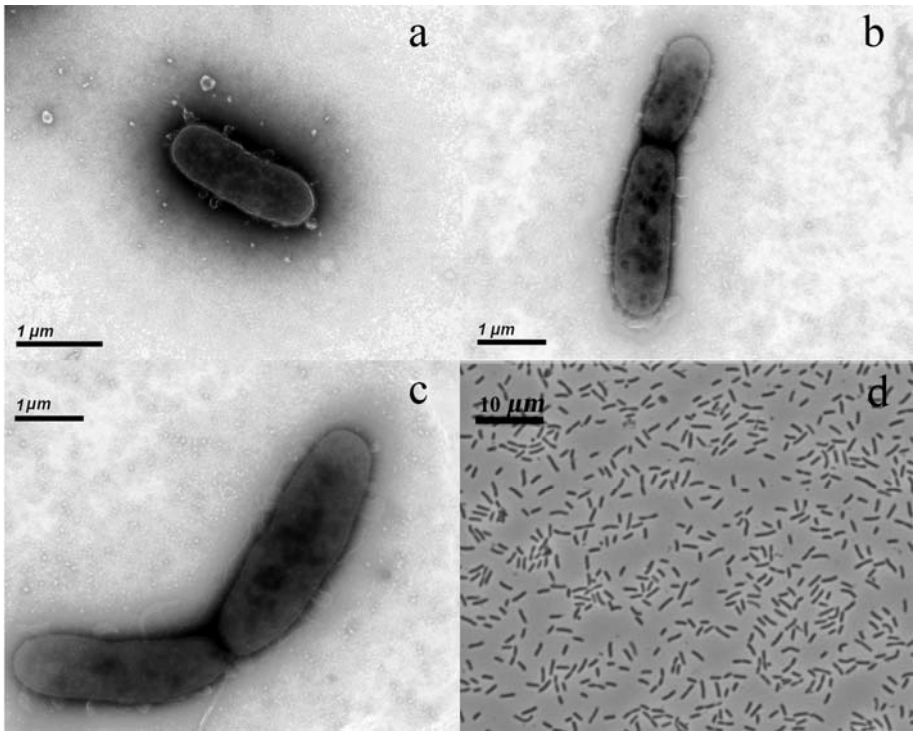


273

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

278

279 **Fig. 2**



280

281 **Fig. 2.** Microscopic images of *Arenibacter nanhaiticus* sp. nov. NH36A^T (bars were shown in each
282 image). a-c, Transmission electron micrographs of negatively stained cells grown on M2 agar
283 medium for 24h at 30 . d, Photomicrograph of vegetative cells viewed by phase-contrast
284 microscopy.

285