Neptunomonas antarctica sp. nov., isolated from marine sediment

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A Gram-negative, motile, oxidase- and catalase-positive and facultatively aerobic bacterium, designated S3-22^T, was isolated from marine sediment of the Nella Fjord, Antarctica. Strain S3-22^T reduced nitrate to nitrite and grew at pH 6.0–8.0, at 4–25 °C and with 0.5–5 % (w/v) NaCl. It contained Q-8 as the only respiratory quinone and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$ as the major cellular fatty acids. The genomic DNA G+C content was 45.6 mol%. Phylogenetic analyses of 16S rRNA gene sequences showed that strain S3-22^T was affiliated with the genus *Neptunomonas*, with 97.1 % sequence similarity to *Neptunomonas japonica* JAMM 0745^T and 94.8 % to *Neptunomonas naphthovorans* NAG-2N-126^T, the type strains of the only two recognized *Neptunomonas* species. DNA–DNA relatedness between strain S3-22^T and *N. japonica* JCM 14595^T was 20.4 %. Strain S3-22^T could be distinguished from the type strains of *Neptunomonas* species by several phenotypic properties. Based on the evidence from our polyphasic study, strain S3-22^T represents a novel *Neptunomonas* species, for which the name *Neptunomonas antarctica* sp. nov. is proposed. The type strain is S3-22^T (=CCTCC AB 209086^T =KACC 14056^T).

The genus *Neptunomonas* was originally proposed by Hedlund *et al.* (1999) and currently contains two species: *Neptunomonas naphthovorans* (Hedlund *et al.*, 1999) and *Neptunomonas japonica* (Miyazaki *et al.*, 2008). The type strain of *N. naphthovorans*, a polycyclic aromatic hydrocarbon-degrading bacterium, was isolated from creosote-contaminated harbour sediment (Hedlund *et al.*, 1999), while that of *N. japonica* was isolated from marine sediment adjacent to a sperm whale carcass (Miyazaki *et al.*, 2008).

A *Neptunomonas*-like bacterial strain, designated S3-22^T, was isolated during a study to screen cold-adapted micro-organisms from Antarctic marine sediment. Samples were collected from the Nella Fjord, Antarctica (69° 22′ 06″ S 76° 22′ 45″ E) at a water depth of 20 m during the 23rd Chinese National Antarctic Research Expedition on 12 January 2007, stored in sterilized plastic bags (250 ml) and transported to the laboratory at 4 °C. For isolation of bacterial strains, approximately 1 g wet sediment was mixed with 99 ml sterilized natural Antarctic seawater supplemented with 10 glass beads (diameter 2–3 mm) and shaken at 4 °C and 300 r.p.m. for 1 h. The suspension was serially diluted (to 10^{-6}) with sterilized seawater and 100 µl aliquots of each dilution were spread onto marine agar 2216 (MA; Difco) plates. After incubation at 4 °C for 4 weeks, individual colonies were picked and purified by subcultivation on MA. Strain S3-22^T was routinely cultivated at 15 °C in TYS broth [0.5 % tryptone (Oxoid), 0.1 % yeast extract (Oxoid), artificial seawater (containing 2.75 % NaCl, 0.07 % KCl, 0.54 % $MgCl_2.6H_2O$, 0.68 % $MgSO_4.7H_2O$, 0.14 % $CaCl_2.2H_2O$, 0.02 % $NaHCO_3$ and distilled water; Miyazaki *et al.*, 2008)] or on TYS agar (1.5 % agar) and was stored at −80 °C in TYS broth with 20 % (v/v) glycerol. *N. japonica* JCM 14595^T was obtained from the Japan Collection of Microorganisms.

Genomic DNA of strain S3-22^T was obtained using a bacterial genomic DNA isolation kit (BioTeke). The 16S rRNA gene of strain S3-22^T was amplified from the

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S3-22^T is FJ713802.

A maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences and a transmission electron micrograph of a negatively stained cell of strain S3-22^T are available as supplementary material with the online version of this paper.

genomic DNA by PCR with the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) (Lane, 1991) and sequenced by Biosune using an automated DNA sequencer (model 3730; Applied Biosystems). The 16S rRNA gene sequence obtained was aligned manually with those of related taxa retrieved from GenBank using MEGA version 4.0 (Tamura *et al.*, 2007). Phylogenetic trees were generated in MEGA using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. The topologies of the phylogenetic trees obtained were evaluated using bootstrap analyses with 1000 replications. Evolutionary distances for the neighbour-joining method were calculated using the model of Jukes & Cantor (1969). The DNA G+C content of strain S3-22^T was determined by HPLC (Mesbah *et al.*, 1989). DNA–DNA hybridization experiments between strain S3-22^T and *N. japonica* JCM 14595^T were conducted at the DSMZ using the methods of De Ley *et al.* (1970) modified by Huß *et al.* (1983).

Cellular morphology was observed by transmission electron microscopy (JEM-100CXII; JEOL) using cells grown in TYS broth for 2 days and negatively stained with 2.5 % (w/v) phosphotungstic acid. Colony morphology was observed after incubation on TYS agar at 15 °C for 10–14 days. The Gram reaction was examined following the standard Gram procedure (Murray *et al.*, 1994). Growth at pH 5.0–9.0 (in increments of 0.5 pH units) was tested at 15 °C in TYS broth with the pH adjusted with MES (pH 5.0–6.0, 50 mM), MOPS (pH 6.5–7.0, 50 mM), Tris (pH 7.5–8.5, 50 mM) or CHES (pH 9.0, 50 mM). Growth at 4, 10, 15, 20, 25, 26, 27, 28 and 30 °C was determined in TYS broth at pH 7.0. Growth with 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 % (w/v) NaCl was examined in broth containing 0.5 % tryptone, 0.1 % yeast extract, 0.5 % $MgCl_2$, 0.2 % $MgSO_4$, 0.05 % $CaCl_2$, 0.1 % KCl, 0.0001 % $FeSO_4$ and distilled water (pH 7.0). Acid production from carbohydrates, including D-glucose, D-fructose, D-mannitol, maltose, D-xylose, D-galactose, sucrose and lactose, was tested at 15 °C using modified O/F medium [0.1 % Casitone (Difco), 0.01 % yeast extract, 0.05 % $(NH_4)_2SO_4$, 0.05 % Tris, 0.001 % phenol red, 1 % carbohydrate, 0.3 % agar and half-strength artificial seawater; Leifson, 1963]. Hydrolysis of starch and casein was tested on TYS agar supplemented with 1 % (w/v) soluble starch or 0.3 % (w/v) casein. Catalase activity was detected by bubble production in 3 % (v/v) H_2O_2 solution. Oxidase activity was determined by using commercial oxidase test strips (Tianhe Microorganism Reagent Co.). DNase activity was detected by using DNase test agar (Tianhe Microorganism Reagent Co.) prepared using artificial seawater. Tests for other enzyme activities, nitrate reduction, aesculin hydrolysis and production of H_2S, indole and acetoin were performed using API ZYM, API 20E and API 20NE strips (bioMérieux) according to the manufacturer's instructions except that cells were suspended in artificial seawater. Anaerobic growth was tested in marine broth 2216 (Difco) using an anaerobic chamber (Forma 1029; Thermo Electron).

After growth in TYS broth at 15 °C for 48 h, cellular fatty acid analysis of cells of strain S3-22^T and *N. japonica* JCM 14595^T was performed at the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing, PR China, according to the instructions of the Sherlock Microbial Identification System. Analysis of respiratory quinones was carried out by Dr Brian Tindall of the DSMZ using the methods described by Tindall (1990a, b).

A nearly full-length 16S rRNA gene sequence of strain S3-22^T (1508 nt) was obtained. Comparison of 16S rRNA gene sequences revealed that strain S3-22^T had highest sequence similarity to the type strains of the two known *Neptunomonas* species (97.1 % to *N. japonica* JAMM 0745^T and 94.8 % to *N. naphthovorans* NAG-2N-126^T) and less than 94 % sequence similarity to strains from other closely related genera. In the neighbour-joining tree, strain S3-22^T was grouped within the phylogenetic branch of the genus *Neptunomonas* with high bootstrap support (98 %; Fig. 1). The maximum-parsimony tree showed a very similar topology (Supplementary Fig. S1, available in IJSEM Online). DNA–DNA relatedness between strain S3-22^T and *N. japonica* JCM 14595^T was 20.4 %. This value is far below the threshold value of 70 % recommended for the delineation of bacterial species (Wayne *et al.*, 1987;

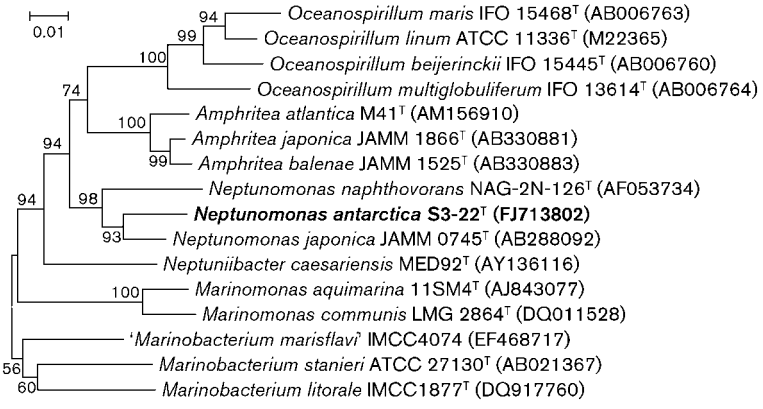

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain S3-22^T and representatives of genus *Neptunomonas* and some other related genera. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

Stackebrandt & Goebel, 1994). Thus, the phylogenetic analysis and DNA–DNA hybridization experiments suggested that strain S3-22^T belongs to the genus *Neptunomonas* and represents a novel species in this genus.

The DNA G+C content of strain S3-22^T was 45.6 mol%, which is an intermediate value between those of *N. japonica* and *N. naphthovorans* (43.6 and 46.3 mol%, respectively). The fatty acids of strain S3-22^T were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 65.6 %), $C_{16:0}$ (17.3 %), $C_{18:1}\omega7c$ (5.5 %), an unknown fatty acid with equivalent chain-length 11.799 (4.3 %) and $C_{10:0}$ 3-OH (4.3 %), which accounted for 97 % of the total cellular fatty acids, and $C_{18:0}$ (0.8 %), $C_{10:0}$ (0.7 %), $C_{12:0}$ (0.7 %), $C_{18:1}\omega9c$ (0.5 %) and $C_{14:0}$ (0.3 %). In comparison with the fatty acid pattern of *N. japonica* JCM 14595^T, strain S3-22^T was similar in that summed feature 3, $C_{16:0}$ and $C_{18:1}\omega7c$ were the major fatty acids (>5 %) and that $C_{10:0}$ 3-OH was the major 3-hydroxy fatty acid, but it was quite different in the proportions of the major fatty acids, especially $C_{18:1}\omega7c$ (Table 1). The only respiratory quinone of strain S3-22^T was Q-8, whereas the other two *Neptunomonas* species possessed minor amounts of Q9 (1 %) in addition to Q-8 (Miyazaki *et al.*, 2008).

The morphological, physiological and biochemical characteristics of strain S3-22^T are given in the species description and in Table 2. Cells of strain S3-22^T were Gram-negative rods, 0.6–1.0 μm in diameter and 1.6–1.8 μm long, that were motile with single polar flagella (Supplementary Fig. S2). Strain S3-22^T exhibited some phenotypic features that are common to the other *Neptunomonas* species, such as facultatively aerobic growth, the presence of oxidase and catalase activities, the absence of amylase activity and the inability to produce H_2S, which supported the phylogenetic assignment of strain S3-22^T to the genus *Neptunomonas*. However, strain S3-22^T could be differentiated phenotypically from the other *Neptunomonas* species by its ability to grow at pH 6. In addition, strain S3-22^T differed from *N. japonica* JCM 14595^T by its ability to produce acids from some carbohydrates and its inability to produce DNase and gelatinase and differed from *N. naphthovorans* by its ability to reduce nitrate and its inability to produce indole or to grow with 7 % NaCl (Table 2).

The results from the phylogenetic analysis of 16S rRNA gene sequences, DNA–DNA hybridization experiments and chemotaxonomic and phenotypic analyses demonstrate that strain S3-22^T represents a novel species of the genus *Neptunomonas*, for which the name *Neptunomonas antarctica* sp. nov. is proposed.

Description of *Neptunomonas antarctica* sp. nov.

Neptunomonas antarctica (an.tarc′ti.ca. N.L. fem. adj. *antarctica* from the Antarctic).

Cells are Gram-negative rods (0.6–1.0 × 1.6–1.8 μm), motile with single polar flagella. Colonies are white, small, circular (0.5–1.2 mm diameter), convex and smooth when grown on TYS agar at 15 °C for 10 days. Facultatively aerobic. Oxidase- and catalase-positive. Growth occurs at 4–25 °C (optimum 15 °C) and pH 6.0–8.0 (optimum pH 6.5–7.0). Grows with 0.5–5 % NaCl (optimum 2–3 %); does not grow without NaCl. Does not hydrolyse casein, aesculin (API 20NE) or starch. Negative for DNase activity. Nitrate is reduced to nitrite (API 20NE). Indole, acetoin (Voges–Proskauer reaction) and H_2S are not produced (API 20E). Acids are produced oxidatively from D-fructose and D-mannitol, but not from D-glucose, maltose, D-xylose, D-galactose, sucrose or lactose. In API 20E tests, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, gelatinase and urease are not detected. In API ZYM tests, alkaline phosphatase, acid phosphatase, leucine arylamidase, esterase (C4) (weak),

Table 1. Fatty acid compositions of strain S3-22^T and the type strains of *Neptunomonas* species

Strains: 1, *Neptunomonas antarctica* sp. nov. S3-22^T; 2, *N. japonica* JCM 14595^T (data from this study); 3, *N. naphthovorans* ATCC 700637^T (Miyazaki *et al.*, 2008). Values are percentages of total fatty acids; fatty acids representing ⩽0.5 % of the total in all strains are not shown. ECL, Equivalent chain-length; −, not detected/not reported.

Fatty acid	1	2	3
$C_{10:0}$	0.7	1.8	−
$C_{10:0}$ 3-OH	4.3	3.4	6.9
$C_{12:0}$	0.7	0.2	6.2
$C_{16:0}$	17.3	10.3	19.5
$C_{16:1}$	−	−	37.8
$C_{18:1}$	−	−	26.5
$C_{18:1}\omega7c$	5.5	21.8	−
Unknown ECL 11.799	4.3	2.1	−
Summed feature 3*	65.6	59.6	−

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

Table 2. Differential characteristics of strain S3-22^T and the type strains of *Neptunomonas* species

Strains: 1, *N. antarctica* sp. nov. S3-22^T; 2, *N. japonica* JCM 14595^T (data from Miyazaki *et al.*, 2008); 3, *N. naphthovorans* ATCC 700637^T (Hedlund *et al.*, 1999). All strains were rod-shaped, Gram-negative, motile, facultatively aerobic, catalase- and oxidase-positive and amylase-negative and negative for H_2S production and contained Q-8 as the major quinone.

Characteristic	1	2	3
Cell dimensions (μm)			
Length	1.2–1.8	1.6–1.8	2.0–3.0
Width	0.6–1.0	0.8–1.0	0.7–0.9
Flagellum position*	P	P/ST	P
Growth with/at:			
7 % NaCl	−	−	+
pH 6	+	−	−
Reduction of nitrate	+	+	−
Production of indole	−	−	+
Acid production from carbohydrates	+	−	+
Enzyme activity			
Gelatinase	−	+	−
DNase	−	+	−
DNA G+C content (mol%)	45.6	43.6	46.3

*P, Polar; ST, subterminal.

esterase lipase (C8) (weak), valine arylamidase (weak) and trypsin (weak) are detected, but lipase (C14), cystine arylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. The respiratory quinone is Q-8. The major cellular fatty acids (>5 %) are summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$. The DNA G+C content of the type strain is 45.6 mol%.

The type strain is S3-22^T (=CCTCC AB 209086^T =KACC 14056^T), isolated from marine sediment of the Nella Fjord, Antarctica.

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