Neptuniibacter halophilus sp. nov., isolated from a salt pan, and emended description of the genus Neptuniibacter

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A bacterial strain designated antisso-13T was isolated from a salt pan in southern Taiwan and characterized using a polyphasic taxonomic approach. Strain antisso-13T was Gram-negative, aerobic, creamy white in colour, rod-shaped and motile by single monopolar flagellum. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain antisso-13T belonged to the genus Neptuniibacter and its closest neighbour was Neptuniibacter caesariensis MED92T, with 96.7 % sequence similarity. Phylogenetic analyses based on rpoB and recA gene sequences and deduced amino acid sequences also revealed that the novel strain and N. caesariensis MED92T formed a distinct phylogenetic cluster. Strain antisso-13T exhibited optimal growth at 25–30 °C, with 2.0–4.0 % NaCl and at pH 7.0. Cellular fatty acids were C16 : 1\(\omega 7c\) (39.8 %), C18 : 1\(\omega 7c\) (29.4 %), C16 : 0 (15.5 %), C10 : 0 3-OH (7.5 %), anteiso-C11 : 0 (1.8 %), C18 : 0 2-OH (1.6 %), iso-C10 : 0 (1.2 %) and C14 : 0 (1.1 %). The major respiratory quinone was ubiquinone Q-8. The polar lipid profile consisted of a mixture of phosphatidylglycerol, phosphatidylethanolamine and several uncharacterized polar lipids and the DNA G+C content was 54.2 mol%. The results of physiological and biochemical tests allowed clear phenotypic differentiation of this isolate from previously described members of the genus Neptuniibacter. It is evident from the genotypic and phenotypic data that strain antisso-13T should be classified as a representative of a novel species in the genus Neptuniibacter. The name proposed for this taxon is Neptuniibacter halophilus sp. nov.; the type strain is antisso-13T (=LMG 25378T=BCRC 80079T). An emended description of the genus Neptuniibacter is provided.

The genus Neptuniibacter, first proposed by Arahal et al. (2007), belongs to the family Oceanospirillaceae of the order Oceanospirillales, class Gammaproteobacteria of the phylum Proteobacteria. At the time of writing this paper, the genus Neptuniibacter comprised only one species with a validly published name, Neptuniibacter caesariensis, which was isolated from a surface seawater sample from the eastern Mediterranean Sea (Arahal et al., 2007). Members of the genus are characteristically Gram-negative, rod-shaped, motile and produce poly-β-hydroxybutyrate granules. Metabolically, Neptuniibacter species are aerobic chemo-organotrophs that prefer organic acids and amino acids as carbon sources and do not ferment carbohydrates. Chemotaxonomically, members of the genus possess Q-8 as the major respiratory quinone, C16 : 1\(\omega 7c\), C18 : 1\(\omega 7c\), C16 : 0, C10 : 0 3-OH and C18 : 0 as the predominant fatty acids, and a DNA G+C content around 47 mol% (Arahal et al., 2007). The aim of the present study was to determine the taxonomic position of a Neptuniibacter-like isolate, antisso-13T, isolated from a salt pan in southern Taiwan.

To determine the taxonomic position, multilocus sequence analysis including the 16S rRNA gene and the genes encoding RNA polymerase beta subunit (rpoB) (Korczak et al. 2004) and recombination repair protein (recA) (Thompson et al., 2005; Sawabe et al., 2007) were analysed. Phylogenetic analyses based on 16S rRNA, rpoB and recA gene sequences revealed that strain antisso-13T belonged to

**Abbreviations:** AL, aminolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, rpoB and recA gene sequences of Neptuniibacter halophilus strain antisso-13T are GQ131677, HM640229 and HM854220, respectively.

Three supplementary figures are available with the online version of this paper.
the genus *Neptuniibacter*, with highest sequence similarity to *N. caesariensis* and the novel strain formed a distinct phylogenetic cluster with *N. caesariensis*.

The sample was collected in a sterile bag from the Cigu Township (Tainan County, southern Taiwan; GPS location 23° 9.6" N 120° 6.16" E), stored at 4 °C and transported to the laboratory within 2–3 h. The sample was homogenized and plated on marine agar 2216 (MA; BD Difco) using a standard dilution plating method. After incubation of the plates at 25 °C for 5 days, strain antiso-13T was purified as a single colony. The strain was preserved at −80 °C as a 20% (v/v) glycerol suspension in marine broth 2216 (MB; BD Difco) or by lyophilization with 20% (w/v) skim milk. *N. caesariensis* MED92T was obtained from the Culture Collection, University of Göteborg (CCUG) and used as reference strain for phenotypic and genotypic tests.

Bacterial cells were observed by light microscopy (DM 2000; Leica) in the lag, exponential and stationary phase of growth to ascertain morphology. Cell motility was tested by the hanging drop and semi-solid agar methods. The Spot Test Flagella Stain (BD Difco) was used for flagellum within the evolutionary radiation occupied by the genus *Neptuniibacter* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain antiso-13T formed a coherent clade with *N. caesariensis* MED92T. Similar topology was obtained in phylogenetic trees generated by the maximum-parsimony and maximum-likelihood algorithms (Fig. 1). According to the pairwise sequence comparisons, strain antiso-13T was closely related to *N. caesariensis* MED92T (96.7% 16S rRNA gene sequence similarity). 16S rRNA gene sequence similarities between strain antiso-13T and other species with validly published names within the order *Oceanospirillales* of the *Gammaproteobacteria* were below 94.8%.

The *rpoB* gene was amplified and sequenced according to Korczak et al. (2004). The sequencing primers used for the *rpoB* gene were *rpoB-F* (5'-GCAGTGAAGARCTCTTG-GTTC-3') and *rpoB-R* (5'-GTTGCAACGACCATT-3') for amplification of a 560 bp fragment from the *rpoB* gene, corresponding to positions 1501–2059 of the *E. coli* *rpoB* gene. The *recA* gene was amplified and sequenced according to Thompson et al. (2005). The sequencing primers used for the *recA* gene were *recA-F* (5'-TGARA-ARCARTYGGTAAGG-3') and *recA-R* (5'-TCRCCANT-TTTRACGCTTACC-3') for amplification of an 840 bp fragment (position 54–898) from the *E. coli* *recA* gene. Phylogenetic trees were constructed as described above but based on the *rpoB* or *recA* genes and deduced amino acid sequences, and they also supported placement of strain antiso-13T within the genus *Neptuniibacter* (see Figs S1 and S2, available in IJSEM Online). A pairwise analysis of the *rpoB* gene sequences and deduced amino acid sequences of strain antiso-13T revealed similarities of 89.4 and 100% with *N. caesariensis* MED92T, respectively. The *rpoB* gene sequence and deduced amino acid sequence
similarities between strain antisso-13T and other species with validly published names within the *Gammaproteobacteria* were below 79.6 and 92.3 %, respectively. According to pairwise *recA* gene sequence and deduced amino acid sequence comparisons, strain antisso-13T was closely related to *N. caesariensis* MED92T with similarities of 84.6 and 98.5 %, respectively. The *recA* gene sequence and deduced amino acid sequence similarities between strain antisso-13T and other species with validly published names within the *Gammaproteobacteria* were below 79.6 and 92.3 %, respectively.

The DNA G+C content of strain antisso-13T was estimated as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by HPLC. The DNA G+C content was calculated from triplicate samples. The DNA G+C content of strain antisso-13T was 54.2 ± 1.0 mol%. Isopenoid quinone was purified by the methods outlined by Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The respiratory quinones of strain antisso-13T were ubiquinone Q-8 (95.0 %) and a minor amount (about 5.0 %) of an unidentified component.

Biomass of antisso-13T and *N. caesariensis* MED92T was obtained after growing the strains on MA at 30 °C for 2 days with aerobic incubation. Fatty acid methyl esters were prepared, separated and identified by using the Instant FAME method of the Microbial Identification System (MIDI) version 6.0 and the RTSBA6.00 database (Sasser, 1990). The fatty acid constituents (>1 %) of strain antisso-13T were C16:1ω7c, C18:1ω7c, C16:0, C10:0 3-OH, anteiso-C11:0, C18:0, C11:0 2-OH, iso-C10:0 and C14:0. The fatty acid profile of strain antisso-13T was similar to that of *N. caesariensis* MED92T, although there were differences in the proportions of some components (Table 1). Like its closest relative *N. caesariensis* MED92T, predominant fatty acids (>5 %) in strain antisso-13T were C16:1ω7c, C18:1ω7c, C16:0 and C10:0 3-OH. However, in contrast to *N. caesariensis* MED92T, strain antisso-13T possessed the following fatty acids (>1 %): iso-C10:0, C11:0 2-OH, anteiso-C11:0 and C14:0 (Table 1).

Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). Molybdophosphoric acid was used for detection of all lipids, ninhydrin reagent was used for lipids containing free amino groups, Zinzadze reagent was used for phosphorus-containing lipids and α-naphthol reagent was used for glycolipids. Strain antisso-13T exhibited a complex polar lipid profile consisting of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), two unidentified aminolipids (AL1, AL2) and an unidentified phospholipid (PL1) (see Fig. S3 Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Neptuniibacter halophilus* strain antisso-13T within the genus *Neptuniibacter*. Numbers at nodes are bootstrap percentages (>70 %) based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Pseudomonas monteilii* CIP 104883T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.
Table 1. Cellular fatty acid composition of Neptuniibacter species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>C10:0 3-OH</td>
<td>7.5</td>
<td>8.6</td>
</tr>
<tr>
<td>iso-C10:0</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>C12:0 2-OH</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>anteiso-C11:0</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>C14:0</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>C16:0</td>
<td>15.5</td>
<td>20.2</td>
</tr>
<tr>
<td>C16:1ω7c</td>
<td>39.8</td>
<td>32.6</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td>C18:1ω7c</td>
<td>29.4</td>
<td>33.5</td>
</tr>
</tbody>
</table>

available in IJSEM Online). Strain antisso-13T exhibited a very similar polar lipid profile to its closest relative and they both had PG, PE, AL1 and PL1. However, AL2 was present in strain antisso-13T but was not found in N. caesariensis MED92T. Two unidentified polar lipids, a glycolipid and PL2, were detected in N. caesariensis MED92T but were not detected in antisso-13T. These results suggest that there are some differences in the polar lipid profiles between the two strains, although they belong to the same genus and have very similar profiles.

Strain antisso-13T and the reference strain were examined for a broad range of phenotypic properties. Activities of catalase, oxidase, DNase and lipase (corn oil, Tweens 20, 40, 60 and 80), hydrolysis of starch and casein, and acetoin production were determined according to standard methods (Smibert & Krieg, 1994). Hydrolysis of alginate [1 % (w/v) sodium alginate] was tested on MA (Hosoya et al., 2009). Hydrolysis of chitin was tested using agar medium supplemented with 0.2 % colloidal chitin (Hsu & Lockwood, 1975). The commercially available API 20NE and API ZYM (both from bioMérieux) kits were used to determine the biochemical properties and enzyme activities of strain antisso-13T and the reference strain. Because strain antisso-13T and the reference strain required seawater-based media for growth, the bacterial sample was suspended in artificial seawater (ASW; NaCl, 24 g; MgCl₂, 5.1 g; Na₂SO₄, 4 g; CaCl₂, 1.1 g; KCl, 0.7 g; NaHCO₃, 0.2 g; KBr, 0.1 g; H₂BO₃, 0.027 g; SrCl₂, 0.024 g; NaF, 0.003 g; distilled water to 1 l; Lyman & Fleming, 1940) for API 20NE and API ZYM tests. Utilization of sugars, alcohols and organic acids as sole carbon and energy sources was tested on basal agar medium [BMA: 50 mM Tris/HCl, pH 7.5, 19 mM NH₄Cl, 0.33 mM KH₂PO₄, 3H₂O, 0.1 mM FeSO₄.7H₂O on ASW solidified with 1.5 % (w/v) Bacto agar; Baumann & Baumann, 1981] containing 0.2 % of the carbon source. Amino acids and amines were tested as sole carbon, nitrogen and energy sources on BMA without NH₄Cl. Compounds were added at 2 g l⁻¹. Growth was monitored for 14 days.

Sensitivity to antibiotics was tested after spreading cells (0.5 McFarland standard) on MA using the following antibiotic discs (μg): ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), nalidixic acid (30), novobiocin (30), penicillin G (10), rifampicin (5), streptomycin (10), sulphamethoxazole (23.75) plus trimethoprim (1.25) and tetracycline (30). The effects of the antibiotics on cell growth were assessed after 3 days of incubation at 30 °C. The diameter of each antibiotic disc is 8 mm and the strain was considered susceptible when the diameter of the inhibition zone was >13 mm, intermediate at 10–12 mm and resistant at <10 mm as described by Nokhal & Schlegel (1983).

The physiological, biochemical and morphological characteristics of strain antisso-13T are given in the species description and Table 2. Phenotypic and genotypic examinations reveal many common traits between the novel strain and its closest relative, N. caesariensis MED92T. Both strains are Gram-negative, rod-shaped, aerobic, motile, chemoheterotrophic, halophilic, mesophilic, and oxidase- and catalase-positive. Poly-β-hydroxybutyrate was accumulated. Their predominant cellular fatty acids are C₁₁₆:₁ω7c, C₁₈:₁ω7c, C₁₆:₀ and C₁₀:₀ 3-OH. The main respiratory quinone of both strains is ubiquinone Q-8. However, strain antisso-13T could be clearly differentiated from N. caesariensis MED92T by a lower NaCl range for growth, by the inability to produce indole, by the inability to hydrolyse Tween 60 and corn oil, by the absence of lipase (C14) and acid phosphatase activities, by the presence of valine arylamidase and cystine arylamidase activities, and by the differences in the utilization of several sugars, organic acids and amino acids as sole carbon, nitrogen and energy sources (Table 2).

On the basis of the 16S rRNA, rpoB and recA gene sequence comparisons, strain antisso-13T occupies a distinct position within the genus Neptuniibacter. This genotypic insight was supported by the unique combination of chemotaxonomic characteristics and biochemical traits of the novel strain (Tables 1 and 2). It is clear from the genotypic and phenotypic data that strain antisso-13T represents a novel species in the genus Neptuniibacter for which the name Neptuniibacter halophilus sp. nov. is proposed.

Emended description of the genus Neptuniibacter
Arahal et al. 2007

The formal descriptions given by Arahal et al. (2007) remain correct, with the addition that although D-glucose is not fermented, some species oxidize D-fructose, D-galactose and D-xylose. Cellular fatty acids (>1%) are C₁₆:₁ω7c, C₁₈:₁ω7c, C₁₆:₀, C₁₀:₀ 3-OH and C₁₈:₀ PG, PE, an unidentified AL and an unidentified PL are present in
Cells are Gram-negative, aerobic, rods (0.2–0.4 μm diameter and 1.5–1.7 μm length) and motile by a single monopolar flagellum. Colonies on MA are creamy white in colour, circular and convex with entire edges. Colonies are approximately 0.2–0.3 mm in diameter on MA after 48 h incubation at 30 °C. Growth occurs at 20–37 °C, 0.5–6.0 % NaCl and pH 7.0–8.0. Optimum growth occurs at 25–30 °C, 2.0–4.0 % NaCl and pH 7.0–7.5. Poly-β-hydroxybutyrate granule accumulation is observed. Positive for oxidase, catalase and DNase activities. Negative for lipase (corn oil) activity and hydrolysis of Tween 20, 40, 60 and 80, casein, starch, alginate and chitin. In API 20NE tests, positive for aesculin hydrolysis and negative for nitrate reduction, indole production, gelatin hydrolysis, D-glucose fermentation, arginine dihydrolase, β-galactosidase and urease activities. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase activities are present and lipase (C14), trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase activities are absent. Growth is positive on succinate, acetate, lactate, L-glutamate, L-alanine, γ-amino-butyric acid, L-aspartate, putrescine, D-fructose, D-galactose, D-xylene, D-saccharate, glycerol, myo-inositol, D-sorbitol, glycin, L-leucine, L-serine, L-threonine, L-arginine, L-ornithine, l-histidine, L-methionine, L-valine, L-lysine and urea, but not on L-cysteine, propionate, citrate, malate, DL-β-hydroxybutyrate, L-arabinose, D-glucose, trehalose, D-mannose, L-rhamnose, maltose, cellulose, sucrose, lactose, melibiose, salicin, D-glucanate, cis-aconitate, N-acetyl-D-glucosamine or D-mannitol. Sensitive to rifampicin, nalidixic acid, kanamycin, chloramphenicol, gentamicin, novobiocin, streptomycin, tetracycline, ampicillin, penicillin G and sulphamethoxazole plus trimethoprim. Cellular fatty acids comprise C₁₆:₁(ω₇c), C₁₈:₁(ω₇c), C₁₆:₀, C₁₀:₀ 3-OH, anteiso-C₁₁:₀, C₁₈:₀, C₁₁:₀ 2-OH, iso-C₁₀:₀ and C₁₄:₀. The major respiratory quinone is ubiquinone Q-8. The polar lipids are PG, PE, two unidentified aminolipids (AL1, AL2) and an unidentified phospholipid (PL1).

The type strain, antisso-13T (=LMG 25378T=BCRC 80079T), was isolated from a salt pan in the Cigu Township, Tainan County, southern Taiwan. The DNA G+C content of the type strain is 54.2 mol%.

**References**


Neptuniibacter halophilus sp. nov.


