Sneathiella chungangensis sp. nov., isolated from a marine sand, and emended description of the genus *Sneathiella*

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A Gram-stain-negative, non-spore-forming, motile, strictly aerobic bacterial strain, designated CAU 1294^T, was isolated from a sand sample and its taxonomic position was investigated using a polyphasic approach. The strain grew optimally at pH 6.5 and 30 °C and in the presence of 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain CAU 1294^T formed a lineage with member of the genus *Sneathiella* and exhibited similarity to *Sneathiella glossodoripedis* MKT133^T (96.3 % similarity) and *Sneathiella chinensis* LMG 23452^T (95.1 % similarity). Strain CAU 1294^T contained Q-10 as the predominant respiratory quinone. $C_{18:1}\omega7c$, $C_{16:0}$ and cyclo-$C_{19:0}\omega8c$ were the major cellular fatty acids. The polar lipids were composed of phosphatidylethanolamine, phosphatidylmethylethanolamine and two unidentified phospholipids. The DNA G+C content was 56.6 mol%. On the basis of these results, strain CAU 1294^T is considered to represent a novel species of the genus *Sneathiella*, for which the name *Sneathiella chungangensis* is proposed. The type strain is CAU 1294^T (=KCTC 32476^T=CECT 8513^T). An emended description of the genus *Sneathiella* is also proposed.

The genus *Sneathiella*, a member of the family *Sneathiellaceae* (Kurahashi *et al.*, 2008), was created by Jordan *et al.* (2007). At the time of writing, this genus consists of two species with validly published names: *Sneathiella chinensis* isolated from coastal sediment (type species; Jordan *et al.*, 2007) and *Sneathiella glossodoripedis* from the foot epidermis of a nudibranch, *Glossodoris cincta* (Mollusca) (Kurahashi *et al.*, 2008). The members of this genus are aerobic, Gram-stain-negative, motile, non-spore-forming, rod-shaped and have Q-10 as the predominant respiratory quinone and $C_{18:1}\omega7c$ as a predominant cellular fatty acid. In the course of screening of bacteria from marine environmental samples, a bacterial strain, designated CAU 1294^T, was isolated from a marine sand sample collected in Jeju Island (33.426866 °N 126.934371 °E) in the Republic of Korea. The purpose of this study was to establish the taxonomic position of this bacterial strain by using a polyphasic characterization that included the determination of phenotypic and chemotaxonomic properties and 16S rRNA gene sequence analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1294^T is KF482756.

Three supplementary figures are available with the online version of this paper.

Isolation was performed according to the protocol of Gordon & Mihm (1962) using marine agar 2216 (MA; Difco), supplemented with cycloheximide (50 mg l^{-1}) and nalidixic acid (20 mg l^{-1}). The crushed sand sample was diluted with sterilized distilled water so that appropriate dilutions could be spread on MA plates. The agar plates were incubated under aerobic conditions at 30 °C for 7 days. A pure single colony was purified by subculturing and preserved at −80 °C in nutrient broth (NB; Difco) supplemented with 25 % (v/v) glycerol. The type strains of two closely related species were used as reference strains in most analyses. *Sneathiella chinensis* NBRC 103408^T was obtained from the NITE Biological Resource Center (NBRC; Chiba, Japan). *Sneathiella glossodoripedis* KCTC 12842^T was obtained from the Korean Collection for Type Cultures (KCTC; Taejon, Korea).

Genomic DNA of strain CAU 1294^T was extracted by the method of Marmur (1961). PCR amplification and sequencing of the 16S rRNA gene of the strain was carried out following established procedures (Cho *et al.*, 2008). The amplified 16S rRNA gene was sequenced directly by using a BigDye Terminator Cycle Sequnceing kit (Applied Biosystems) and an automatic model 3730 DNA sequencer (Applied Biosystems). Multiple alignments and calculation

of the levels of 16S rRNA gene sequence similarity with those sequences of type strains of species of the genus *Sneathiella* and a broad selection of the closely related genera were performed by using the EzTaxon-e server (Kim *et al.*, 2012; http://eztaxon-e.ezbiocloud.net/) and CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distance matrices were generated by the neighbour-joining method described by Jukes & Cantor (1969). Phylogenetic trees were generated using the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981) algorithms in the PHYLIP package (Felsenstein, 1989). Branch support in the neighbour-joining tree was evaluated by the bootstrap resampling method, with 1000 replicates (Felsenstein, 1985). The nearly complete 16S rRNA gene sequence of strain CAU 1289^T (1519 bp) was determined and compared with the corresponding sequences of other bacterial strains in the public database. Phylogenetic analysis indicated that the strain represented a member of the genus *Sneathiella*. The neighbour-joining tree is shown in Fig. 1. The trees obtained with the maximum-likelihood and least-squares algorithms showed basically the same topology (Fig. S1a, b, available in the online Supplementary Material). Pairwise analysis showed that the most closely related strains were *Sneathiella glossodoripedis* MKT133^T (16S rRNA gene similarity, 96.3 %) and *Sneathiella chinensis* LMG 23452^T (similarity, 95.1 %).

For the investigation of morphological, physiological and biochemical characteristics, strain CAU 1294^T was cultivated on MA at 30 °C. Cell morphology was investigated under a light microscope (model DM 1000; Leica) and a transmission electron microscope (JEM 1010; JEOL) using cells from an exponentially growing culture. For transmission electron microscopy, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Gram staining was carried out using the bioMérieux Gram staining kit according to the manufacturer's instructions. Motility was assessed using the hanging-drop method and transmission electron microscopy. Growth between 4 °C and 40 °C in an aerobic incubator (model MIR-253; Sanyo) and in an anaerobic chamber (Bactron; Sheldon) was determined by measuring the turbidity of marine broth 2216 (MB; Difco) after 72 h of incubation. The pH range for growth was investigated in MB that had been adjusted to pH 4.5–10.0 (at intervals of 0.5 pH units) by using 3 M sodium acetate/1 M acetic acid and 3 M Na_2CO_3 buffers. Growth in the absence of NaCl and in the presence of 0–15.0 % (w/v) NaCl was investigated in trypticase soy broth (TSB) prepared

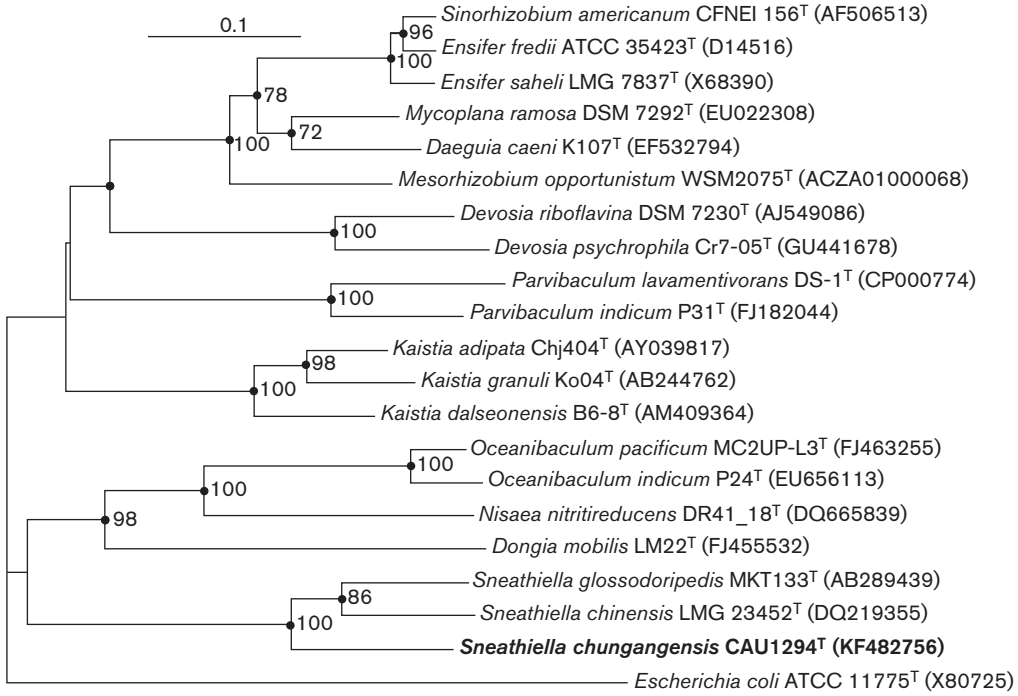

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 1294^T and the type strains of species of the genus *Sneathiella* with validly published names. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and least-squares algorithms. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >70 % are given. Bar, 0.1 substitutions per nucleotide position. *Escherichia coli* ATCC 11775^T (X80725) is used as an outgroup organism.

according to the formula of the Difco medium except that NaCl was excluded and 0.45 % (w/v) $MaCl_2 . 6H_2O$ or 0.06 % (w/v) KCl was added. Oxidase activity was evaluated from the oxidation of 0.1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Catalase activity was determined from bubble production in 3 % (v/v) H_2O_2 solution. Hydrolysis of casein, starch and urea were determined according to the methods of Lányí (1987) and Smibert & Krieg (1994). Acid production from carbohydrates, enzyme activity and other physiological and biochemical features were tested as described by Leifson (1963), using API 20E and API ZYM strips (bioMérieux) according to the manufacturer's instructions (with an incubation time of up to 5 days at 30 °C).

The morphological, cultural, physiological and biochemical characteristics of strain CAU 1294^T are shown in Table 1 or given in the species description. Overall, the results obtained in this study are in agreement with previously published data for the two species of the genus *Sneathiella*. However, strain CAU 1289^T differed from its closest relatives, *Sneathiella glossodoripedis* KCTC 12842^T (Kurahashi *et al.*, 2008) and the type strain of the type species of the genus *Sneathiella*, *Sneathiella chinensis* NBRC 103408^T (Jordan *et al.*, 2007) in its colony colour, growth requirements, acetoin production and weak activities of alkaline phosphatase and naphthol-AS-BI-phosphohydrolase. Despite the fact that CAU 1294^T was isolated from a saline environment, it did not require NaCl for growth, though it was shown to tolerate NaCl concentrations of up to 6 % (w/v).

For the determination of fatty acid composition, cell masses of strain CAU 1294^T, *Sneathiella glossodoripedis* KCTC 12842^T and *Sneathiella chinensis* NBRC 103408^T were harvested from TSA (Difco) after cultivation for 5 days at 30 °C. The physiological age of the biomasses harvested for fatty acid analysis was standardized by observing growth development during incubation of the three different cultures and choosing the moment of harvesting according to the standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Cellular fatty acid methyl esters were obtained by using the method of Minnikin *et al.* (1980) and separated in a model 6890N gas chromatograph (Agilent) fitted with a model 7683 autosampler (Agilent). Peaks were identified by using the Microbial Identification software package (MOORE library version 5.0; MIDI database TSBA6). Menaquinones were separated by HPLC using an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] with a flow rate of 1 ml min^{-1} (Komagata & Suzuki, 1987). The polar lipids of strain CAU 1294^T were identified using two-dimensional TLC by the method of Minnikin *et al.* (1984). The plate was sprayed with 10 % ethanolic molybdatophosphoric acid (for the total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids) or α-naphthol/sulphuric acid reagent (for glycolipids) (Sigma-Aldrich). The mol% G+C content of the genomic DNA was determined using HPLC by the method of Tamaoka & Komagata (1984) with the modification that the DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

Table 1. Differential properties of strain CAU 1294^T and the type strains of species of the genus *Sneathiella*

Strains: 1, CAU 1294^T (data from this study); 2, *Sneathiella chinensis* NBRC 103408^T (data from this study except for the DNA G+C content, taken from Jordan *et al.*, 2007); 3, *Sneathiella glossodoripedis* KCTC 12842^T (data from this study except for the DNA G+C content and cell size, taken from Kurahashi *et al.*, 2008). +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3
Colony colour	Yellow	Cream	Cream
Growth at 45 °C	−	−	+
Growth without NaCl	+	+	−
Acetoin production	+	−	−
Enzyme activity			
Alkaline phosphatase	w	+	+
Naphthol-AS-BI-phosphohydrolase	w	w	+
DNA G+C content (mol%)	56.6	57.1	56.9

Strain CAU 1294^T contained Q-10 as a predominant quinone. This characteristic is in agreement with results observed previously for *Sneathiella glossodoripedis* KCTC 12842^T (Kurahashi *et al.*, 2008). The fatty acid profile was very similar to those of type strains of the species of the genus *Sneathiella*. The strain contained unsaturated, straight-chain, branched and hydroxy (Table 2). The major fatty acids of strain CAU 1294^T were $C_{18:1}\omega7c$ (33.4 %), $C_{16:0}$ (24.1 %) and cyclo-$C_{19:0}\omega8c$ (15.4 %), which are characteristic of the type strain of the type species of the genus, *Sneathiella chinensis* NBRC 103408^T, but cyclo-$C_{19:0}\omega8c$ was not found in *Sneathiella glossodoripedis* KCTC 12842^T. The following fatty acids are present at a level of at least 1 %: $C_{12:0}$, $C_{17:1}\omega6c$, $C_{14:0}$ 3-OH, $C_{16:1}\omega7c$, $C_{18:1}\omega5c$ and $C_{19:0}\omega8c$. However, some quantitative differences in fatty acid content could be observed between strain CAU 1294^T and two species of the genus *Sneathiella*. The polar lipids were composed of phosphatidylethanolamine, phosphatidylmethylethanolamine and two unidentified phospholipids (Fig. S2). The genomic DNA of strain CAU 1294^T had a G+C content of 56.6 mol%. These data provide sufficient evidence to support the proposal to recognize strain CAU 1294^T as representing a novel species of the genus *Sneathiella*, for which the name *Sneathiella chungangensis* sp. nov. is proposed.

Emended description of the genus *Sneathiella* Jordan *et al.* 2007

The description is as given by Jordan *et al.* (2007) with the following amendments. The major polar lipids are phosphatidylethanolamine and phosphatidylmethylethanolamine. Unidentified phospholipids also occur.

Table 2. Cellular fatty acid composition (%) of strain CAU 1294^T and the type strains of species of the genus *Sneathiella*

Strains: 1, CAU 1294^T; 2, *Sneathiella chinensis* NBRC 103408^T; 3, *Sneathiella glossodoripedis* KCTC 12842^T. All data were taken from this study. −, not detected.

Fatty acid	1	2	3
Hydroxy			
$C_{13:0}$ 3-OH	−	−	0.3
$C_{14:0}$ 3-OH	4.9	5.0	4.3
Straight-chain			
$C_{9:0}$	−	−	0.2
$C_{10:0}$	−	−	1.1
$C_{12:0}$	7.4	−	0.08
$C_{14:0}$	1.6	2.3	1.5
$C_{16:0}$	24.1	20.9	17.2
$C_{17:0}$	0.7	0.4	0.8
$C_{18:0}$	0.1	0.1	0.2
Unsaturated			
$C_{15:1}\omega8c$	−	0.8	0.4
$C_{15:1}\omega6c$	−	0.2	−
$C_{16:1}\omega11c$	0.3	1.2	−
$C_{16:1}\omega7c$	1.2	12.5	4.9
$C_{16:1}\omega5c$	0.8	2.6	1.2
$C_{17:1}\omega8c$	0.4	0.6	1.1
$C_{17:1}\omega6c$	6.6	8.2	15.0
$C_{18:1}\omega7c$	33.4	28.5	49.1
$C_{18:1}\omega5c$	1.3	2.5	0.5
Branched			
iso-$C_{17:1}\omega9c$	−	−	0.6
cyclo-$C_{19:0}$ $\omega8c$	15.4	12.7	−
Methyl			
Summed Feature 2*	0.3	0.3	0.2

*Summed features consist of two or more fatty acids that could not be separated by GLC using the MIDI system. Summed feature 2 comprised $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I.

Description of *Sneathiella chungangensis* sp. nov.

Sneathiella chungangensis (chung.ang.en′sis. N.L. fem. adj. *chungangensis* belonging to Chung-Ang University where the taxonomic studies on this species were performed).

Cells are Gram-stain-negative, non-spore-forming, strictly aerobic rods approximately 0.3–0.5 μm in diameter and 0.9–1.2 μm in length, motile by means of a single polar flagellum (Fig. S3). Colonies on MA are yellowish, circular, convex and 0.5–1.0 mm in diameter after 5 days of incubation at 30 °C. Growth occurs at 20 °C–37 °C (optimum, 30 °C) and at pH 5.5–8.0 (optimum, pH 6.5). Growth occurs in the presence of 0–6 % (w/v) NaCl [optimum, 2 % (w/v) NaCl]. Catalase, oxidase and production of acetoin are positive. Production of H_2S and nitrate reduction are negative. Starch, gelatin, casein and urea are not hydrolysed. Citrate is not utilized. D-Glucose, D-mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose are not assimilated. In assays with the API ZYM system, acid phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, alkaline phosphatase, esterase (C4) and esterase lipase (C8) are detected. The predominant isoprenoid quinone is Q-10. The predominant cellular fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ and cyclo-$C_{19:0}\omega8c$. The polar lipid pattern consists of phosphatidylethanolamine, phosphatidylmethylethanolamine and two unidentified phospholipids.

The type strain is CAU 1294^T (=KCTC 32476^T=CECT 8513^T), which was isolated from a marine sand collected in Jeju Island in the Republic of Korea. The DNA G+C content of the type strain is 56.6 mol%.

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