Sneathiella glossodoripedis sp. nov., a marine alphaproteobacterium isolated from the nudibranch *Glossodoris cincta*, and proposal of *Sneathiellales* ord. nov. and *Sneathiellaceae* fam. nov.

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A novel marine bacterium, designated strain MKT133^T, was isolated from the foot epidermis of a nudibranch, *Glossodoris cincta* (Mollusca), collected in seawater off the coast of Japan at a depth of 4 m. This bacterium was Gram-negative, motile, mesophilic and strictly aerobic, with small rod-shaped cells. Colonies of the strain after 4–5 days incubation on marine agar 2216 at 30 °C were less than 1 mm in diameter. The strain required salt for growth and contained Q-10 as the predominant respiratory quinone, C_{18:1}ω7c, C_{16:0} and C_{17:1} as major cellular fatty acids and C_{14:0} 3-OH as a hydroxy fatty acid. 16S rRNA gene sequence analysis showed that the isolate had highest similarity to *Sneathiella chinensis*, with 97.2% sequence similarity to the type strain. Our phylogenetic analysis also revealed that this clade represents a distinct lineage and forms a deep branch with less than 90% 16S rRNA gene sequence similarity to the members of the eight known orders within the *Alphaproteobacteria*. Sufficient differences exist to distinguish this strain from *Sneathiella chinensis*. The name *Sneathiella glossodoripedis* sp. nov. is proposed, with the type strain MKT133^T (=IAM 15419^T =KCTC 12842^T). The novel order *Sneathiellales* ord. nov. and family *Sneathiellaceae* fam. nov. are proposed for the distinct phyletic line represented by the genus *Sneathiella*.

The monophyletism of the class *Alphaproteobacteria* is widely supported by a number of phylogenetic analyses (Garrity *et al.*, 2005), and the taxonomic name has been published in Validation List no. 107 (Garrity *et al.*, 2006). Recent investigations, however, have indicated that the species belonging to the class *Alphaproteobacteria* are quite diverse in their phenotypic characteristics. Many species isolated from marine environments are classified in the classes *Alphaproteobacteria* and *Gammaproteobacteria*, and the numbers of bacteria of these classes are growing quickly. The objective of our study is to focus on the diversity of bacteria in gastrointestinal tracts and attached internal organs and on the epidermis of marine organisms (Kurahashi & Yokota, 2002). We describe herein a novel bacterium that represents a distinct lineage and that forms a deep branch with *Sneathiella chinensis* in the *Alphaproteobacteria*. *Sneathiella chinensis* was reported as a novel genus and species in the class *Alphaproteobacteria*

(Jordan *et al.*, 2007) and was considered to be a member of the order *Rhizobiales*. However, the present study indicates that *Sneathiella chinensis* with strain MKT133^T should be reclassified as members of a new order and family in the class *Alphaproteobacteria*.

Isolate MKT133^T was obtained from the foot epidermis of a nudibranch (*Glossodoris cincta*; Mollusca) collected in the sea off Aka island (Okinawa, Japan) from a depth of approximately 4 m, using MG plates [per litre distilled water: 37.4 g marine broth 2216 (Becton Dickinson), 6.0 g gellan gum]. Standard methods were used for isolation and phenotypic characterization, as described previously (Kurahashi & Yokota, 2004). Cultivation was performed on a marine agar 2216 (MA) plate at 30 °C for 1 week.

Colony morphology, size and colour were examined from cultures grown aerobically at 30 °C for 1 week on MA plates and R2AMN agar plates [per litre distilled water: R2A agar (Becton Dickinson), 18.2 g; MgCl₂·6H₂O, 10.0 g; MgSO₄·7H₂O, 6.0 g; NaCl, 3.0 g]. Salt tolerance was tested in R2AM medium [per litre distilled water: R2A

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MKT133^T is AB289439.

agar, 18.2 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.0 g] supplemented with 0–8 % (w/v) NaCl after incubation for 1 week at 30 °C. The Gram reaction was determined using the Becton Dickinson Gram stain kit, following the manufacturer's instructions. General cell morphology was studied using phase-contrast microscopy and transmission electron microscopy (TEM). Specimens for TEM were negatively stained with phosphotungstic acid. Biochemical tests were carried out using the API 20E and API ZYM microbial identification strips (bioMérieux). Results are given in the species description.

Strain MKT133^T was Gram-negative. Cells were rods, 0.2–0.3 × 0.7–1.0 µm, slightly curved and motile in broth medium. One polar flagellum was present on negatively stained whole-cell preparations. Spore formation was not observed, and intracellular granules were detected in the cells. No growth was detected on R2A agar supplemented with only NaCl, but was observed with the further addition of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Colonies on MA plates were colourless, circular, convex, smooth and less than 1.0 mm in diameter after incubation for 1 week at 30 °C; compared with this, on R2AM medium supplemented with 3 % (w/v) NaCl, colonies became slightly yellowish and were larger (1.5–2.0 mm). No growth was detected under anaerobic conditions, even with prolonged incubations of 30 days. The isolate grew at 17–45 °C, with optimum growth around 40 °C. Strain MKT133^T could grow in the presence of up to 4 % (w/v) NaCl, and no growth was observed in medium containing no NaCl, while growth of *S. chinensis* occurred without NaCl (Jordan *et al.*, 2007). Phenotypic characteristics that differentiate strain MKT133^T from *S. chinensis* are summarized in Table 1. These divergences demonstrate that strain MKT133^T represents a distinct species.

For the initial identification of whole-cell fatty acids, cells were grown for 1 week at 30 °C on MA. Cellular fatty acid

methyl esters were prepared and analysed using gas chromatography according to the instructions of the Microbial Identification System (MIDI). Subsequently, to identify a mixed signal peak, separation of hydroxy and non-hydroxy fatty acid methyl esters was performed by TLC using n-hexane/diethyl ether (1 : 1, v/v). Each fraction was analysed again by the MIDI system. Respiratory quinones were extracted with chloroform/methanol (2 : 1, v/v), fractionated by a spin column and identified by LC-MS using an LCMS-QP8000 α (Shimadzu). The G + C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). DNA–DNA hybridization was performed to evaluate the genomic DNA relatedness between strain MKT133^T and *S. chinensis* LMG 23452^T. DNA–DNA hybridization experiments were carried out fluorometrically, using photobiotin-labelled DNA probes and microdilution wells as described by Ezaki *et al.* (1989). The temperature of renaturation was 48.3 °C for 18 h.

The cellular fatty acid composition of MKT133^T was dominated by $\text{C}_{18:1}\omega 7\text{c}$ (54.1 %), $\text{C}_{16:0}$ (13.6 %), $\text{C}_{17:1}\omega 6\text{c}$ (12.0 %) and $\text{C}_{14:0}$ 3-OH (4.2 %) (Table 2). Strain MKT133^T and *S. chinensis* have very similar fatty acid profiles, considering the fact that fatty acids can be transformed biosynthetically (e.g. $\text{C}_{18:1}\omega 7\text{c} \rightarrow \text{cyclo C}_{19:0}\omega 8\text{c}$) by varying the culture conditions slightly. Many members of the *Alphaproteobacteria* are characterized by the dominant fatty acids $\text{C}_{18:1}\omega 7\text{c}$ and $\text{C}_{16:0}$, indicating that the fatty acid data for MKT133^T and *S. chinensis* are consistent with a phylogenetic position within the class *Alphaproteobacteria*. However, the presence of large amounts of both $\text{C}_{17:1}$ and $\text{C}_{14:0}$ 3-OH in these two strains is a distinct feature from other members of the *Alphaproteobacteria*. Detection of the quinone system Q-10 at a peak area ratio of approximately 93 % is in agreement with the phylogenetic affiliation within the *Alphaproteobacteria*. The DNA G + C content of strain MKT133^T (56.9 mol%) is almost the same as that of the reference strain *S. chinensis* CBMAI 737^T (57.1 mol%). The DNA–DNA relatedness between strain MKT133^T and *S. chinensis* LMG 23452^T was about 22 %, clearly below the 70 % threshold generally accepted for species delineation (Stackebrandt *et al.*, 2002).

The 16S rRNA gene was amplified using universal primers as described previously (Kurahashi & Yokota, 2004), and PCR products were sequenced directly in both directions. The sequences were analysed with the FASTA search system (Pearson & Lipman, 1988) on the DDBJ website to find closely related bacterial 16S rRNA gene sequences. The sequence of the isolate was aligned with all GenBank entries. Alignment was performed using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were inferred by neighbour-joining (Saitou & Nei, 1987) with Kimura's two-parameter model (Kimura, 1980) and maximum-parsimony (Fitch, 1971) with a heuristic search. The topology of the phylogenetic tree was evaluated by using Felsenstein's bootstrap resampling method (Felsenstein, 1985) with 1000 replicates.

Table 1. Comparison of properties between strain MKT133^T and *S. chinensis* LMG 23452^T

Data for *S. chinensis* LMG 23452^T were taken from Jordan *et al.* (2007) unless indicated. ND, No data available.

Characteristic	Strain MKT133 ^T	<i>S. chinensis</i> LMG 23452 ^T
Cell size (µm)	0.2–0.3 × 0.7–1.0	ND
Growth without NaCl	–	+
Growth at 45 °C	+	–
Reduction of nitrite to N ₂	–	+
Production of acetoin	+	–
Arginine dihydrolase	+	–
Ornithine decarboxylase	+	–
Urease	–	–, +
G + C content (mol%)	56.9	57.1

*Data from this study.

Table 2. Cellular fatty acid compositions (%) of strain MKT133^T and *S. chinensis* LMG 23452^T

Fatty acid	Strain MKT133 ^T	<i>S. chinensis</i> LMG 23452 ^T	
		This study	Jordan <i>et al.</i> (2007)
Hydroxy			
C _{13:0} 3-OH	0.5	0.5	—
C _{14:0} 3-OH	4.2	6.6	2.7
Straight-chain			
C _{9:0}	0.2	0.2	—
C _{10:0}	1.0	1.1	—
C _{12:0}	0.1	0.1	—
C _{14:0}	0.8	1.0	0.8
C _{15:0}	3.1	—	2.1
C _{16:0}	13.6	15.4	17.2
C _{17:0}	0.9	1.0	0.5
C _{18:0}	0.3	0.8	—
Unsaturated			
C _{15:1} ω8c	0.4	0.2	0.4
C _{15:1} ω6c	0.1	—	—
C _{16:1} ω11c	—	—	1.4
C _{16:1} ω7c	2.8	2.7	7.0
C _{16:1} ω5c	1.0	0.8	1.7
C _{17:1} ω8c	1.5	0.9	0.7
C _{17:1} ω6c	12.0	9.4	5.6
C _{18:1} ω7c	54.1	56.2	46.2
C _{18:1} ω5c	0.7	0.7	0.9
Branched			
iso-C _{17:1} ω9c	0.3	0.2	—
cyclo C _{19:0} ω8c	—	—	9.8
Methyl			
11-Methyl	—	—	0.3
C _{18:1} ω7c	—	—	—
Unknown	0.9	1.8	1.1
Summed feature 2*	0.8	0.3	1.7
Summed feature 4*	0.3	—	—
Summed feature 7*	0.4	0.4	—

—, Not detected during analysis.

*Summed features: 2, C_{12:0} (aldehyde?); 4, iso-C_{17:1} I/anteiso-C_{17:1} B; 7, unknown equivalent chain-length 18.846/C_{19:1}ω6c.

The FASTA search with the 16S rRNA gene sequence of MKT133^T showed that the newly determined sequence was related to those of the *Alphaproteobacteria*. The closest relatives of strain MKT133^T with validly published names were *Sneathiella chinensis* and *Kordiimonas gwangyangensis*, with 97.2 and 89.2 % sequence similarity to the respective type strains. Type strains of other species belonging to the *Alphaproteobacteria* such as *Mesorhizobium loti* (89.2 %), *Silicibacter lacuscaerulensis* (88.5 %), *Asticcacaulis excentricus* (88.2 %), *Parvularcula bermudensis* (88.1 %), *Sphingomonas paucimobilis* (88.8 %), *Rickettsia prowazekii* (86.6 %) and *Tistrella mobilis* (89.0 %) showed less than 90 % 16S rRNA gene sequence similarity. The phylogenetic position of the new isolate based on the 16S rRNA gene

sequence is shown in Fig. 1. The phylogenetic tree suggested that the isolate formed a separate cluster with *S. chinensis* and two clones, as indicated by the 100 % bootstrap value, thus creating a novel taxon. *S. chinensis* was considered to be a member of the order *Rhizobiales* by Jordan *et al.* (2007) but, in this study, the resultant phylogenetic tree revealed that *S. chinensis* forms a new phyletic lineage with strain MKT133^T distinct from all eight orders of the class *Alphaproteobacteria*. The class *Alphaproteobacteria* presently contains the orders *Caulobacteriales*, *Kordiimonadales*, *Parvularculales*, *Rhizobiales*, *Rhodobacteriales*, *Rhodospirillales*, *Rickettsiales* and *Sphingomonadales*, and segregates on a distinct, monophyletic 16S rRNA gene tree branch (Garrity *et al.*, 2005; Kwon *et al.*, 2005). Accordingly, it might be reasonable to suppose that the genus *Sneathiella* should be reclassified as a member of a new order.

On the basis of morphological and physiological characteristics, fatty acid profile, DNA G+C content and DNA–DNA relatedness, strain MKT133^T can be considered to be a member of the genus *Sneathiella*. However, there were some differences between strain MKT133^T and *S. chinensis* in their genetic and phenotypic characteristics. The phylogenetic distinctiveness was sufficient to categorize strain MKT133^T as a member of a species that is distinct from *S. chinensis*. The divergent phylogenetic relationships between the genus *Sneathiella* and established orders in the *Alphaproteobacteria* supported the creation of a new order and family within the class *Alphaproteobacteria*. On the basis of these results, strain MKT133^T is proposed as the type strain of a novel species of the genus *Sneathiella*, *Sneathiella glossodoripedis* sp. nov., in a new order and family.

Description of *Sneathiellales* ord. nov.

Sneathiellales (Snea.thi.el.la'les. N.L. fem. n. *Sneathiella* type genus of the order; -ales ending to denote an order; N.L. fem. n. *Sneathiellales* the order of *Sneathiella*).

Segregation of these organisms into a new order is justified by their distinct phyletic lineage based on the 16S rRNA gene. The type genus is *Sneathiella*.

Description of *Sneathiellaceae* fam. nov.

Sneathiellaceae (Snea.thi.el.la'ceae. N.L. fem. n. *Sneathiella* type genus of the family; -aceae ending to denote a family; N.L. fem. n. *Sneathiellaceae* the family of *Sneathiella*).

Description is the same as that for the order. The type genus is *Sneathiella*.

Description of *Sneathiella glossodoripedis* sp. nov.

Sneathiella glossodoripedis [glos.so.do.ri.pe'dis. N.L. n. *Glossodoris* name of a zoological genus; L. gen. n. *pedis* of the foot; N.L. gen. n. *Glossodoripedis* of the foot of

Gram-negative. Cells are slightly curved rods, 0.2–0.3 × 0.7–1.0 µm, motile by one polar flagellum, non-spore-forming and have poly-β-hydroxybutyrate inclusions. Colonies on MA are colourless, circular, convex, smooth and less than 1.0 mm in diameter after incubation for 1 week at 30 °C. Optimum growth occurs at 40 °C, while no growth occurs at 10 or 50 °C. Cultures grow aerobically, but not anaerobically. The ubiquinone system is Q-10 and the major cellular fatty acids are C_{18:1ω7c}, C_{16:0}, C_{17:1} and C_{14:0} 3-OH. No growth occurs without NaCl. The DNA G+C content of the type strain is 56.9 mol%. The type strain is positive for catalase, oxidase, arginine dihydrolase, ornithine decarboxylase, tryptophan deaminase, production of acetoin, utilization of citrate and reduction of nitrate to nitrite, but negative for urease, gelatinase, production of H₂S and indole, reduction of nitrite to N₂ and assimilation of D-glucose, D-mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. In assays with the API ZYM system, alkaline phosphatase, esterase (C4) (weak), esterase lipase (C8) (weak), leucine arylamidase, trypsin (weak), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase

The type strain is MKT133^T (=IAM 15419^T =KCTC 12842^T), which was isolated from the nudibranch *Glossodoris cincta* off Aka island, Okinawa, Japan.

We thank Dr Jean P. Euzéby (Ecole Nationale Vétérinaire, Toulouse, France) for his help in the latinization of the new species name. This work was supported in part by a Grant-in-Aid for Scientific Research (no. 17310135) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Y.F. and S.H. thank the New Energy and Industrial Technology Development Organization (NEDO) for financial support.

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