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Sunxiuqinia dokdonensis sp. nov., Isolated from Deep Sub-Seafloor Sediment[§]

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A novel facultatively anaerobic strain DH1[T] was isolated from deep sub-seafloor sediment at a depth of 900 m below the seafloor off Seo-do (the west part of Dokdo Island) in the East Sea of the Republic of Korea. The new strain was characterized using polyphasic approaches. The isolate was Gram-stain-negative, motile by gliding, non-spore-forming rods, oxidase-negative, and catalase-positive; and formed colonies of orange-red color. The NaCl range for growth was 0.5–7.0% (w/v) and no growth was observed in the absence of NaCl. The isolate grew optimally at 30°C, with 2% (w/v) NaCl and at pH 7. The cell-wall hydrolysates contained ribose as a major sugar. The DNA G+C content was 40.8 mol%. The closest related strains are *Sunxiuqinia faeciviva* JAM-BA0302[T] and *Sunxiuqinia elliptica* DQHS-4[T] (97.9 and 96.3% sequence similarity, respectively). The level of DNA-DNA relatedness between strain DH1[T] and *S. faeciviva* JAM-BA0302[T] was around 41% (but only 6% between DH1[T] and *S. elliptica* DQHS-4[T]). The major cellular fatty acids of the isolate were contained iso-$C_{15:0}$ (25.9%), anteiso-$C_{15:0}$ (16.7%), and summed feature 9 (comprising $C_{16:0}$ 3-OH and/or unknown fatty acid of dimethylacetal ECL 17.157; 13.2%). The predominant menaquinone was MK-7. On the basis of polyphasic evidence from this study, the isolate was considered to represent a novel species of the genus *Sunxiuqinia*, for which the name *Sunxiuqinia dokdonensis* sp. nov. is proposed; the type strain is DH1[T] (=KCTC 32503[T] =CGMCC 1.12676[T] =JCM 19380[T]).

Keywords: *Sunxiuqinia dokdonensis*, 16S rRNA, taxonomy, Dokdo, Sub-seafloor, *Bacteroidetes*

Introduction

Very diverse array of prokaryotes inhabit marine subsurface sedimentary environments, comprising a great portion of the total living biomass on Earth (Whitman *et al.*, 1998; D'Hondt *et al.*, 2009). In spite of this great phylogenetic diversity of microorganisms in the deep sea environment, only a few novel microorganisms have been isolated from deep sub-seafloor sediments and validly described (Bale *et al.*, 1997; Mikucki *et al.*, 2003; Toffin *et al.*, 2004; Kendall *et al.*, 2006). Some of the strains isolated from sub-seafloor sediments showed characteristics distinguishing them from previously reported species, and most belong to the phylum *Bacteroidetes* (Batzke *et al.*, 2007; Kobayashi *et al.*, 2008; Parkes *et al.*, 2009). The genus *Sunxiuqinia*, which was first described by Qu *et al.* (2011), is also a member of the phylum *Bacteroidetes*. At the time of writing, there were only two recognized species of the genus *Sunxiuqinia*. These two isolates are *S. elliptica* isolated from sediment of a seashore pond used for sea cucumber culture in Jimo, Qingdao, on the east coast of China (Qu *et al.*, 2011) and *S. faeciviva* isolated from deep sub-seafloor sediment at a depth of 247.1 m below the seafloor off the Shimokita Peninsula of Japan (Takai *et al.*, 2013).

A culturable facultative bacterial strain was isolated from deep sub-seafloor sediment off the coast of Dokdo Island, in the East Sea of the Republic of Korea and it was determined to be related to the genus *Sunxiuqinia* on the basis of 16S rRNA gene sequence analysis. Using polyphasic approaches, the exact taxonomic position of the new isolate was determined in this study.

Materials and Methods

Sampling and isolation

A 0.25-m^2 boxcore was deployed at a depth of 900 m below the seafloor off Dokdo Island (37°14′29″N 131°51′38″E) in the East Sea of the Republic of Korea. Immediately on retrieval, the sediments with seawater were transferred into N_2-flushed Duran bottles (1 L). Before sealing the bottle, 20 ml of 2.5% (w/v) cysteine hydrochloride (Yakuri) and 2.5% (w/v) sodium sulfide (Sigma, USA) were added to the Duran bottle to keep a strict anaerobic condition. The samples were stored in a refrigerator at 4°C before use. For the isolation and purification of the anaerobic deep-sea bacteria, a novel anaerobic jar with the capacity to bear high pressure was used for cultivation (Lee *et al.*, 2013b). The strict anaerobic marine agar 2216 (MA; Difco, USA) and broth (MB; Difco) were prepared and sterilized using the anaerobic technique

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described by Hungate (1969). Resazurin (1 mg/L final concentration, Sigma) was added to the anaerobic MA or MB media for indication of O_2. A colony was newly isolated from a strict anaerobic MA in a pressurized anaerobic jar (Lee *et al.*, 2013b) with 100% N_2 gas phase. The isolate was anaerobically purified on MA at 30°C in a Coy anaerobic chamber (Grass Lake, USA) by more than five rounds of single colony isolation. After the isolate was found to be able to grow under both aerobic and anaerobic growth conditions, it was routinely cultivated on MA under aerobic condition and maintained on plates of MA (pH 7.0), as lyophilized cultures at 4°C and in 6% (v/v) dimetyl sulfoxide (DMSO) at -80°C.

Strains

S. faeciviva JAM-BA0302^T (=JCM 15547^T) and *S. elliptica* DQHS4^T (=CGMCC 1.9156^T) were obtained from the JCM and the CGMCC culture collections, respectively, and were used as reference strains for comparison in this study.

Phylogenetic analysis

An almost-complete part of the 16S rRNA gene of DH1^T was amplified using the universal primers 27F and 1492R (Cho and Giovannoni, 2003), and was sequenced by Solgent Co. Ltd, in Daejeon, Korea. Sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) under the following thermal cycling conditions: 30 cycles of 96°C for 10 sec, 52°C for 5 sec and 60°C for 4 min. Nucleotide sequences were determined in an ABI PRISM 3730XL DNA analyzer by Solgent. The almost complete sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR, USA). The 16S rRNA gene sequences of the reference strains used for phylogenetic analysis were obtained from the EzTaxon-e (http://eztaxon-e.ezbiocloud.net; Kim *et al.*, 2012) or GenBank (Benson *et al.*, 1993) database. Sequence similarities were calculated using the EzTaxon-e server. Multiple alignment with closely related sequences was performed using CLUSTAL W (Thompson *et al.*, 1997). A phylogenetic tree was drawn using the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood (Felsenstein, 1981) methods using MEGA 5.2 software (Tamura *et al.*, 2011). Robustness of tree topologies was evaluated by bootstrap analysis based on 1,000 re-samplings (Felsenstein, 1985).

G+C content and DNA-DNA hybridization

Highly purified genomic DNA from each 2 L culture of *S. faeciviva* JCM 15547^T, *S. elliptica* CGMCC 1.9156^T, and the DH1^T strain was extracted as previously described (Lee *et al.*, 2013a). The G+C mol% of highly purified each genomic DNA from *S. elliptica* CGMCC 1.9156^T, *S. faeciviva* JCM 15547^T, and strain DH1^T was assessed by the method of Mesbah *et al.* (1989) using a YMC-Triat C_{18} column (150×4.6 mm) as previously described (Lee *et al.*, 2013a). In order to make sure that the new isolate DH1^T is truly a novel species of the genus *Sunxiuqinia*, DNA-DNA hybridization analysis was performed. The DNA-DNA hybridization was carried out at 42°C (calculated with correction for the presence of 50% formamide) using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989).

Morphological, physiological, and biochemical characterization

Cell morphology, size, presence of flagella, and Gram staining were examined by light, phase contrast (Nicon DS-Ri1), and transmission electron microscopy. Transmission electron microscopy of negatively stained cells was carried out as described by Zillig *et al.* (1990) and examined under a CM 20 (Philips, Netherlands) electron microscope. Flagellation was also checked by the staining method previously described by Heimbrook *et al.* (1989). Gliding motility was investigated as described by Bowman (2000). Gram-staining was determined using a Difco Gram-staining set (Gerhardt *et al.*, 1994). Commercial MA and MB were not used for tests for utilization of carbohydrates and proteinaceous substrates. Instead, a modified MA or MB in which both yeast extract and peptone were substituted with 0.5% (w/v) test substrate was used. The temperature range for growth was determined on MA at 4, 10, 15, 20, 25, 30, 35, 37, 40, and 45°C. Marine broth (MB) which contained no NaCl was prepared and the growth range of NaCl on this modified MB supplemented with 0–12% NaCl (at intervals of 0.5% NaCl; at 30°C) was assessed for seven days. Turbidity was monitored at OD_{600} using a microplate reader (Model 680; Bio-Rad, USA). The pH range for growth was determined in MB over the pH range of 4–10 at intervals of 0.5 pH unit. The following buffer systems were used for pH tests: Citrate/Na_2HPO_4 (pH 4–6), phosphate buffer (pH 7–8), Na_2CO_3/$NaHCO_3$ (pH 9–10) as described by Gomori (2010). Catalase activity was examined by bubble production in a 3% (v/v) aqueous hydrogen peroxide solution, and oxidase activity was determined by Oxy-swab (bioMérieux, France). Physiological and biochemical characteristics were examined using API 20 NE, API 50CH, and ZYM test strips (bioMérieux) according to the manufacturer's instructions. After inoculation, API 20 NE strips were incubated at 30°C for 2 days. API 50CH strips were incubated for 2 weeks at 30°C. API ZYM strips were incubated for 4 h at 30°C.

Chemotaxonomic characterization

For fatty acid analysis, a loop of cell mass was collected from MA after cultivation for 4 days at 30°C and the cellular fatty acids from each cell were obtained and quantified as previously described (Lee *et al.*, 2013a) by a GC system (Model 6890N and 7683 autosampler; Agilent, USA) according to a standard protocol (Sherlock Microbial Identification System; MIDI). The fatty acids were identified using the Microbial Identification software package (Moore 150 library). Polar lipids were extracted and their patterns obtained as previously described (Tindall, 1990a, 1990b; Lee *et al.*, 2013a). Polar lipids were extracted from freeze-dried cell material (50 mg) using a two-stage method and separated by two-dimensional silica-gel TLC. Patterns for polar lipids were obtained by spotting samples on the corner of thin layer plates (silica gel 60 pre-prepared plates of Merck Art. No. 5554, 20 × 20 cm). The plates were developed with chloroform/methanol/water (65:25:4, v/v) in the first direction and chloroform/acetic acid/methanol/water (80:12:15:4, v/v) in

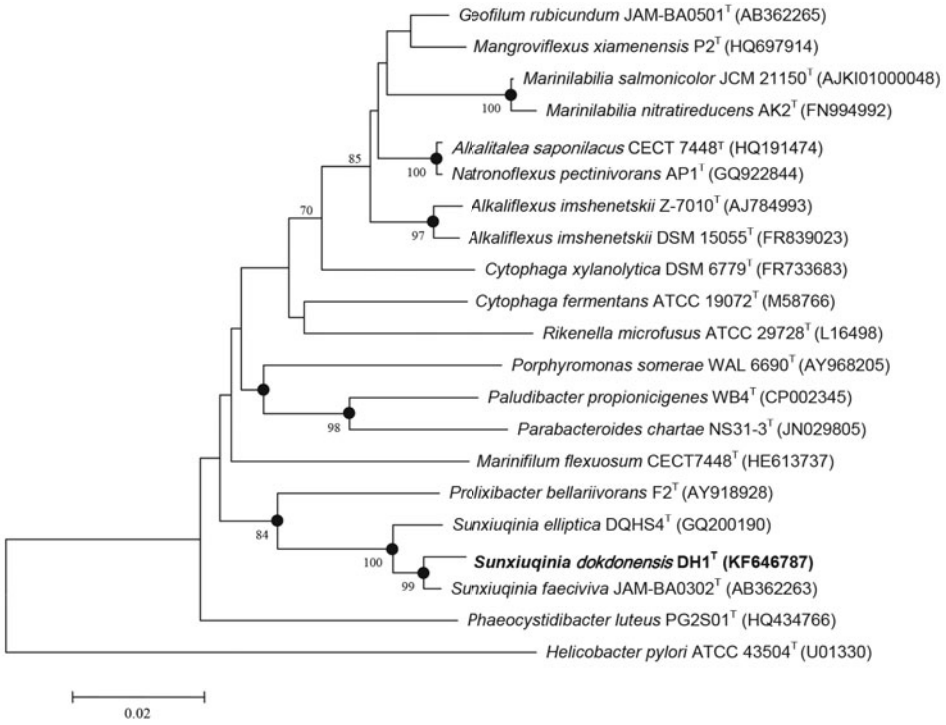

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogentic position of strain DH1^T and representative members of the phylum *Bacteroidetes*. Bootstrap values (expressed as percentages of 1,000 replication, >70%) are shown at branching points. Dots indicate that the corresponding branches are also present in the maximum-likelihood and the max-imum-parsimony tree. *Helicobacter pylori* ATCC 43504^T was used as an outgroup. Bar, 0.02 substitution per nucleotide position.

the second direction. Whole-cell sugars were determined by TLC as described previously by Becker *et al.* (1965).

Results and Discussion

Phylogenetic analysis

Strain DH1^T showed the closest sequence similarity to *S. faeciviva* JAM-BA0302^T (97.9%) and *S. elliptica* DQHS4^T (96.3%). The next nearest one was *Prolixibacter bellariivorans* F2^T (90.2%). A phylogenetic tree was constructed (Fig. 1) and it indicated that strain DH1^T belonged to the genus *Sunxiuqinia* and should be assigned to the genus *Sunxiuqinia.*

G+C content and DNA-DNA hybridization

The DNA G+C content of strain DH1^T was 40.8 mol%, which was the lowest among the tested *Sunxiuqinia* species (Table 1). DNA-DNA hybridization values between DH1^T and *S. faeciviva* JCM 15547^T, and *S. elliptica* CGMCC 1.9156^T were 41±3% and 6±2%, respectively. Based on the cut-off value of 70% (Wayne *et al.*, 1987) for discriminating bacterial species, the new isolate DH1^T should be considered a representative of a novel species of the genus *Sunxiuqinia*.

Morphological, physiological, and biochemical characterization

No flagella were observed using either transmission electron microscopy (Fig. 2) or phase contrast microscopy (data not shown). Gliding motility was exhibited. Morphological, cultural, physiological and biochemical characteristics of strain DH1^T are listed in Table 1. All the data shown in Table 1 were obtained in this study by cultivating *S. elliptica* CGMCC 1.9156^T, *S. faeciviva* JCM 15547^T, and strain DH1^T. Strain DH1^T was quite different from *S. elliptica* CGMCC 1.9156^T based on growth response to O_2, cell shape and other many physiological characteristics (Tables 1 and 2). However, strain DH1^T showed clear phenotypic similarities to *S. faeciviva*

Table 1. Different phenotypic characteristics of strain DH1^T and closely related species of the genus *Sunxiuqinia*. Species: 1, *Sunxiuqinia* sp. DH1^T; 2, *S. faeciviva* JCM 15547^T; 3, *S. elliptica* CGMCC 1.9156^T. All data were from this study. All the phenotypic characteristics of strain DH1^T determined by API tests are detailed in the species description. +, Positive reaction; –, negative reaction. FA, Facultatively anaerobic; SA, strictly aerobic; SCR, straight to curved rod; E, elliptical.

Characteristics	1	2	3
Growth response to O_2	FA	FA	SA
Cell shape	SCR	SCR	E
Temperature range (°C)	25-37	20-35	15-40
pH range	6-8	6-8	6-8.5
NaCl range % (w/v)	0.5-7	0.5-6	0.5-10
Utilization of:			
Yeast extract	-	+	+
Tryptone	-	+	+
Casamino acid	-	-	+
API 20NE :			
Indole	-	-	+
API ZYM :			
Leucine arylamidase	-	-	+
α-Chymotrypsin	-	+	+
α-Glucosidase	-	-	+
α-Fucosidase	-	-	+
API 50CH (acid production) :			
D-Lyxose	-	-	+
DNA G+C content(mol%)	40.8	43.8	41.8

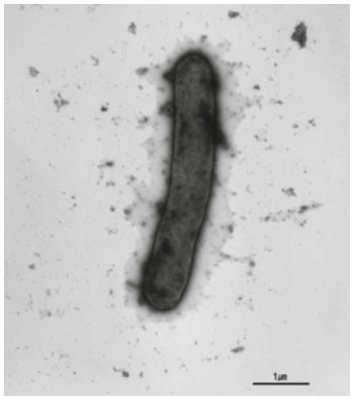

Fig. 2. Transmission electron micrograph of strain DH1^T grown on MA at 30°C for 3 days. Bar, 1 um.

JCM 15547^T, including cell morphology, orange-red pigment, facultative anaerobic growth, and narrow pH range (pH 6–8) for growth. Both strains showed no growth at pH≤5.5 and ≥8.5. However, growth of strain DH1^T at 7% (w/v) NaCl was observed while growth of *S. faeciviva* JCM 15547^T was not, which is consistent with a previous report (Takai *et al.*, 2013). Another clear difference was that DH1^T was not able to utilize yeast extract and tryptone in the modified MB, which was used by *S. faeciviva* JCM 15547^T. All the detailed phenotypic characteristics of strain DH1^T determined by API tests are shown in Table 1 and detailed in the species description.

Table 2. Cellular fatty acid composition of strain DH1^T and related type strains of the genus *Sunxiuqinia*. Species: 1, *Sunxiuqinia* sp. DH1^T; 2, *S. faeciviva* JCM 15547^T; 3, *S. elliptica* CGMCC 1.9156^T. All strains were grown on MA (Difco 2216) at 30°C for 4 days. FAME, Fatty acid methyl ester; DMA, dimethyl acetal; ALDE, aldehyde; tr, trace (<1%); -, not detected.

Fatty acids	1	2	3
$C_{15:0}$ FAME	-	2.6	tr
$C_{16:0}$ FAME	3.8	4.2	1.6
$C_{17:0}$ FAME	-	4.7	3.2
$C_{14:0}$ DMA	2.7	-	tr
$C_{15:1}$ *cis* 9/t 8 FAME	2.9	5.9	1.8
$C_{17:1}$ *cis*-11 FAME	-	5.2	6.2
$C_{16:1}$ *cis*-9 FAME	-	2.9	tr
iso-$C_{15:0}$ FAME	25.9	14.8	17.6
anteiso-$C_{15:0}$ FAME	16.7	13.4	10.5
iso-$C_{17:0}$ FAME	-	-	5.9
iso-$C_{16:0}$ FAME	6.2	5.8	5.3
anteiso-$C_{17:0}$ FAME	-	-	1.8
iso-$C_{14:0}$ FAME	2.2	2.1	1.5
iso-$C_{13:0}$ FAME	-	-	tr
iso-$C_{15:0}$ DMA	-	1.4	-
anteiso-$C_{17:0}$ 3OH FAME	9.2	8.4	7.5
iso-$C_{15:0}$ 3OH FAME	5.6	4.3	4.2
$C_{17:0}$ 3OH FAME	-	2.2	1.2
Summed feature[a]	1.7	0.9	4.5
3	1.7	0.9	4.5
6	-	2.0	tr
9	13.2	11.7	3.9
11	9.8	7.9	19.5

[a] Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 comprised iso-$C_{15:0}$ ALDE and/or unknown fatty acid of ECL 13.570, summed feature 6 comprised anteiso-$C_{15:0}$ 3-OH FAME and/or $C_{16:1}$ *cis*-7 DMA, summed feature 9 comprised iso-$C_{16:0}$ 3-OH FAME and/or unknown fatty acid of ECL 17.157 DMA, summed feature 11 comprised iso-$C_{17:0}$ 3-OH FAME and/or $C_{18:2}$ DMA.

Chemotaxonomic characterization

The most abundant fatty acid of both strains DH1^T and *S. faeciviva* JCM 15547^T was iso-$C_{15:0}$ but the amount of iso-$C_{15:0}$ was almost doubled in strain DH1^T (26%) compared to *S. faeciviva* JCM 15547^T (15%) (Table 2). In addition, $C_{17:0}$ dimmethylacetal (DMA) (3%) was only detected in strain DH1^T. The complete fatty acid composition of strain DH1^T and two other reference strains are given in Table 2. The polar lipid profile of DH1^T consisted of the compound phophatidylethanolamine, an unknown glycolipid, two unknown phospholipids, and three unknown lipids. Polar lipid profile was similar to *S. faeciviva* JCM 15547^T (Supplementary data Fig. S1). Both strains (DH1^T and *S. faeciviva* JCM 15547^T) contained ribose as a major component of cell-wall sugar, but glucose was also detected as a minor component from strain DH1^T. In contrast, glucose was determined to be a major, and ribose as a minor, component in the cell-wall of *S. elliptica* CGMCC 1.9156^T (Supplementary data Fig. S2).

Taxonomic conclusion

Strain DH1^T shared 97.9% 16S rRNA gene sequence similarity and showed 41% DNA-DNA hybridization with *S. faeciviva* JCM 15547^T clearly demonstrating that the strain represents a new species of the genus *Sunxiuqinia* (Qu *et al.*, 2011). The isolate can be differentiated from *S. faeciviva* JCM 15547^T by its phenotypic and chemotaxonomic characteristics (Tables 1 and 2). Overall, molecular phylogenetic and physiological data clearly distinguished strain DH1^T from the other two validly described *Sunxiuqinia* species. Therefore, DH1^T should be considered to represent a novel species; for which the name *Sunxiuqinia dokdonensis* is proposed.

Description of *Sunxiuqinia dokdonensis* sp. nov.

Sunxiuqinia dokdonensis [dok.do.nen'sis. N.L. fem. adj. *dokdonensis* of Dokdo, the island from where the type strain was isolated].

Cells are Gram-stain-negative, motile by gliding, straight to curved rods (4–28 μm length in the exponential growth phase) and no flagella. Facultatively anaerobic. Catalase-positive but oxidase-negative. Colonies on MA are convex, circular, orange-red in color and 1–2 mm diameter after 4 days at 30°C. Grows at 25–37°C (optimum 30°C), at pH 6–8 (optimum pH 7) and with 0.5–7.0% (w/v) NaCl (optimum, 2%). In API 20NE tests, Production of β-galactosidase and β-glucosidase are positive but reduction of nitrate to nitrite, indole production, gelatin hydrolysis, arginine dihydrolase, and urease are negative. The type strain does not assimilate D-glucose, D-mannose, L-arabinose, malic acid, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, trisodium citrate, and phenylacetic acid. In API 50CH tests, positive for esculin, and 5-keto-gluconate and negative for all other tests. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), naphtol-AS-

BI-phosphohydrolase, and N-acetyl-β-glucosaminidase activities are present; the other enzyme activities are absent. The major cellular fatty acids (>10%) are iso-$C_{15:0}$ (25.9%), anteiso-$C_{15:0}$ (16.7%), and summed feature 9 (comprising $C_{16:0}$ 3-OH and/or unknown fatty acid of ECL 17.157; 13.2%). The major respiratory quinone is MK-7 and the polar lipids are phosphatidylethanolamine, an unknown glycolipid, two unknown phospholipids, and three unknown lipids. The DNA G+C content is 40.8 mol%.

The type strain is DH1^T (=KCTC 32503^T =CGMCC 1.12676^T =JCM 19380^T), isolated from deep sub-seafloor sediment at a depth of 900 m below the seafloor off Seo-do, part of Dokdo Island (37°14′ 29″ N 131° 51′38″ E), in the East Sea of the Republic of Korea.

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