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Abyssisolibacter fermentans gen. nov. sp. nov., isolated from deep sub-seafloor sediment[§]

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A Gram-staining-negative, thin rod-shaped, anaerobic bacterium designated MCWD3^T was isolated from sediment of the deep sea in Ulleung Basin, East Sea, Korea. The ranges of temperature, pH and NaCl for growth of this strain were 15–40°C (optimum 29°C), 5.0–10.0 (optimum pH 6.5), and 1–5%, respectively. The major fatty acids were iso-$C_{15:0}$ (30%) and iso-$C_{15:0}$ dimethyl acetal (17%). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and unidentified aminophospholipids, phospholipids, and aminolipids. The fermentation product from yeast extract was acetate. Phylogenetic analysis based on 16S rRNA genes indicated that the isolate was related to *Sporosalibacterium faouarense* (92.8% sequence identity), *Clostridiisalibacter paucivorans* (92.6%), and *Brassicibacter mesophilus* (92.4%). However, the isolate was differentiated from these genera by both physiological and chemotaxonomical properties. On the basis of a polyphasic taxonomic analysis, we propose that MCWD3^T represents a novel taxon with the name *Abyssisolibacter fermentans* gen. nov. sp. nov.

Keywords: polyphasic taxonomy, *Clostridiaceae*, deep marine sediment, *Abyssisolibacter fermentans* gen. nov. sp. nov.

Introduction

Diverse microbes are present in the various deep seabed environments despite the extreme conditions of low temperature, high pressure and low nutrient availability (Jørgensen and Boetius, 2007). Recent ocean drilling projects have provided more insights into the microbial systems in the deep-sea sediments. Analyses based on 16S rRNA gene sequences have uncovered specific microbiomes (Zhu *et al*., 2013; Polymenakou *et al*., 2015) as well prokaryotes phylogenetically distant from those in other habitats (Marchesi *et al*., 2001; Reed *et al*,. 2002). While these investigations have provided more information about the deep-sea microbiome, full understanding for deep sea microbes is still limited by the scarcity of cultivated strains and information about their properties (Teske, 2006; Da Silva *et al*., 2013).

The order Clostridiales, is a large and diverse phylogenetic group that includes species with a wide range of morphologies, habitats and physiologies (Cato *et al*., 1986). The family *Clostridiaceae* belongs to this order and contains 37 genera as well as a number of unclassified species (http://www.ncbi.nlm.nih.gov/Taxonomy/). In the last decade, a number of isolates have been described that represent novel genera in this family (Liebgott *et al*., 2008; Rezgui *et al*., 2011; Fang *et al*., 2012; Hania *et al*., 2015a). We isolated another member of the family *Clostridiaceae* from deep-sea sediment in Ulleung Basin, East Sea, Korea. A polyphasic analysis of its growth properties, chemotaxonomy, and phylogeny demonstrated that this isolate represents a novel species and genus in the family.

Materials and Methods

Isolation and growth of strain MCWD3^T

Strain MCWD3^T was isolated from the deep sea sediment (water depth 2,150 m) located in the Ulleung Basin (37°00′N, 131°00′E). The samples were acquired by a 4 m long piston core sampler with polycarbonate liner (ϕ=13 cm). Subsamples were obtained from 0.5, 50, 100, 150, and 200 cm depths by a modified 3 ml plastic syringe. The subsamples, 0.5 cm^3, were mixed with 10 ml of anaerobic medium (DSMZ No. 135) on shipboard. Following transportation to the laboratory, 0.5 ml of culture broth was transferred into a new medium (10 ml) composed of (g/L): K_2HPO_4 0.14, NH_4Cl 0.5, $FeSO_4$ 0.017, KCl 0.34, $MgCl_2{\cdot}6H_2O$ 2.8, $MgSO_4{\cdot}7H_2O$ 3.45, $CaCl_2{\cdot}2H_2O$ 0.14, NaCl 22, trace mineral solution (Balch, 1979) 1 ml, vitamin solution (Balch, 1979) 1 ml, resazurin 1 mg, yeast extract 2, glucose 5, sodium acetate 1.4, cysteine·H_2O·HCl 0.5, $NaHCO_3$ 5.0, $Na_2S{\cdot}9H_20$ 0.5. The sample was diluted and the enrichment was performed at 30°C for 7 days. The highest dilution showing growth was streaked into a slant of the same medium with 1.5% agar. A resulting colony was selected for further characterization. It was preserved as a glycerol suspension (15%, w/v) at –80°C and deposited at KCTC (Korean Collection for Type Cultures, No. KCTC 15524) and CCTCC (under deposit). All of media for this study were prepared by the Hungate anaerobic techniques (Hungate, 1969). Unless otherwise stated, strain MCWD3^T was cultivated at 30°C.

Phylogenetic analysis

Extraction of genomic DNA from strain MCWD3^T was con-

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ducted using a commercial Exgene cell SV kit (GeneAll Biotechnology), and the 16S rRNA gene was amplified with bacterial primers 27F and 1492R. The PCR product was sequenced at Cosmogentech Inc. The GenBank accession number is KU510224. To ascertain the phylogenetic position of strain MCWD3^T, the 16S rRNA gene sequence was compared with those obtained from EzTaxon (http://eztaxon-e.ezbiocloud.net/). A total of 1,343 unambiguously aligned sequences were compared. Phylogenetic tree using the Jukes-Cantor model and neighbor-joining (NJ) method was constructed by the MEGA ver. 5.2 (Tamura *et al.,* 2011). The resulting tree topologies were evaluated using bootstrap analyses based on 1,000 resamplings. Phylogenetic analysis based on maximum-likelihood (ML) and maximum-parsimony (MP) methods were also conducted in MEGA.

Morphological, physiological, and biochemical characterization

Gram type was examined by utilizing Gram staining kit (YD diagnostics). Morphology and motility were observed by light microscopy at × 1,000 (Nikon). The oxygen resistance was assessed by cultivation with air in the absence of Na_2S and cysteine. To examine growth temperature ranges, cultures were incubated at 4, 10, 15, 20, 25, 29, 33, 37, 40, and 44°C. The pH range for was determined at pH values of 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 10.0 by the addition of 10 mM of buffer. The buffers and pH ranges use were by MES (pH 4.0–6.0), HEPES (pH 6.0–8.0) and AMPSO (pH 8.0–10.0) (Yang *et al*., 2013) instead of bicarbonate buffer. Salinity range for growth was examined by the addition of NaCl to final concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 10.0%. The effect of yeast extract on growth was tested at 0, 0.05, 0.1, and 0.2%. Endospore formation was checked by the pasteurization test, and the strains were treated at 80°C, 90°C and 100°C for 10 or 20 minutes before being transferred into fresh medium (Hania *et al*., 2015b).

Type strain *Sporosalibacterium faouarense* SOL3f37^T (Rezgui *et al*., 2011) was obtained from KCTC (No. 15135) and cultivated with the medium (No. 987) recommended by the institute.

The basal medium for the substrate utilization tests for strain MCWD3^T was prepared with (g/L): K_2HPO_4 0.3, KH_2PO_4 0.3, NH_4Cl 1.0, $MgCl_2{\cdot}6H_2O$ 2.0, KCl 0.1, $CaCl_2{\cdot}2H_2O$ 0.1, NaCl 10.0, trace mineral solution 10 ml, yeast extract 0.5, resazurin 0.1% (w/v), $NaHCO_3$ 0.2, cysteine-HCl 0.5 (pH 6.5). The organic acids (20 mM) tested were sodium formate, sodium acetate, sodium propionate, sodium butyrate, sodium fumarate, sodium malate, sodium succinate, sodium lactate, and sodium citrate. The amino acids (20 mM) tested were alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The carbohydrates (20 mM) tested were arabinose, cellobiose, fructose, glucose, glycerol, lactose, maltose, mannose, and mannitol. In addition, H_2+CO_2 gas (80:20 v:v, 1 bar), peptone (0.1%), and casamino acids (0.1%) were tested. The basal medium for *Sporosalibacterium faouarense* SOL3f37^T was prepared with the previous composition (Rezgui *et al*., 2011) containing 0.05% yeast extract.

To examine if sulfur compounds can be served as electron acceptors, potassium sulfate (20 mM), sodium thiosulfate (20 mM), and elemental sulfur (0.1%) were added into each yeast extract (0.05%) containing medium prepared in the absence of Na_2S. Hydrogen sulfide concentration of each sample was photometrically estimated from colloidal CuS (Cord-Ruwisch, 1985). The reduction of nitrate and nitrite was inspected by utilizing Durham tubes (Ogg and Patel, 2009) and color changes with the Griess reagents (Conn and Breed, 1919; MacFaddin, 2000). The end product from the metabolism was confirmed by high pressure liquid chromatography (HPLC) with an ion exclusion coloumn (Shodex RSpak KC81), which was operated at a flow rate of 1.0 ml/min and 40°C.

Chemotaxonomy

The cellular fatty acids from strain MCWD3^T and *Sporosalibacterium faouarense* SOL3f37^T were analyzed by the MIDI/Hewlett Packard Microbial Identification System (MIS: Sasser, 1990) with Sherlock ver. 6.1 and ANAER6 database accord-

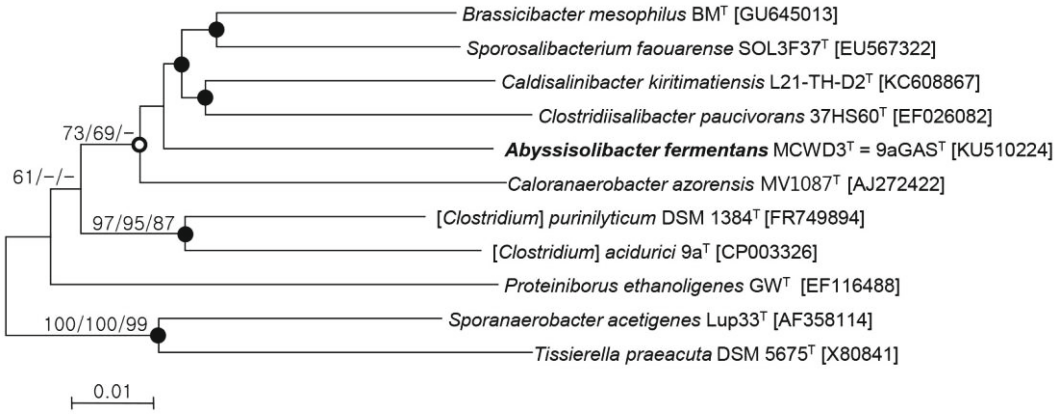

Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationship between strain MCWD39^T and related members of the family *Clostridiaceae*. The tree is based on the Juke & Cantor distances model and the neighbour-joining algorithm. Bootstrap values (>50% from 1,000 replicates) from NJ, ML, and MP are placed on left of the node. Scale bar, 0.01 nucleotide substitutions per nucleotide position. Filled circles represents nodes recovered in 3 different treeing methods, and hollow circles represent node recovered in 2 methods.

ing to the manufacturer's instruction with cells cultivated for 7 days. Polar lipids were extracted as described by Minnikin *et al.* (1984) and analyzed by 2-dimensional thin-layer chromatography (TLC; Minnikin *et al.*,1984; Komagata and Suzuki, 1987). To stain the total lipids, phospholipid, aminolipid, glycolipid and complex lipid, molybdophosphoric acid, molybdenum blue, ninhydrin, and α-naphthol and sulfuric acid were sprayed, respectively. Detailed procedure was described in Yang *et al.* (2013).

Results and Discussion

Phylogenetic analysis

The 16S rRNA gene sequence of strain MCWD3^T revealed that it was affiliated with the family *Clostridiaceae* of the order *Clostridiales*. The most closely related strains were *Sporosalibacterium faouarense* SOL3F37^T (92.7% sequence identity; Rezgui *et al.*, 2011), *Clostridiisalibacter paucivorans* 37HS60^T (92.6%; Liebgott *et al.*, 2008), and *Brassicibacter mesophilus* BM^T (92.4%; Fang *et al.*, 2012). In the phylogenetic tree constructed by the neighbor-joining (NJ) method, strain MCWD3^T grouped within the clade containing the genera *Sporosalibacterium, Clostridiisalibacter*, *Brassicibacter*, *Caloranaerobacter,* and *Caldisalinibacter* (Fig 1). The analysis also indicated that strain MCWD3^T was not closely affiliated with any other previously described genus.

Morphology and physiological characteristics

Strain MCWD3^T stained Gram-negative and formed thin, long rods (0.7 μm × 5-23 μm). Under the microscope, cells were motile. The colonies were yellow, circular and smooth. No growth was observed in air, indication that it was a strict anaerobe. Growth occurred at 15–40°C (optimum 29°C) and pH 4.0–10.0 (optimum pH 6.5). NaCl was required at range of 1–5% NaCl (w/v, optimum 2%) for growth (Table 1). The pasteurization test resulted in successful re-growth even after incubation at 100°C for 20 min, indicating thermoresistant endospores were formed.

Yeast extract was required for growth even in the presence of other carbon sources. Casamino acids and peptone did not stimulate growth. The colorimetric test indicated that

Table 1. Differential characteristics of strain MCWD3^T and phylogenetically closely related species. Strains 1. MCWD3^T, 2. *Sporosalibacterium faouarense* SOL3f37^T (Rezgui *et al.*, 2011), 3. *Brassicibacter mesophilus* BM^T (Fang *et al.*, 2012), 4. *Clostridiisalibacter paucivorans* 37HS60^T (Liebgott *et al.*, 2008), 5. *Caloranaerobacter azorensis* MV1087^T (Wery *et al.*, 2001), 6. *Caldisalinibacter kiririmariensis* L21-TH-D2^T (Hania *et al.*, 2015a). All microbes in the table are motile. Substrate utilization assays for strains 1 and 2 were from this study. The characteristics of *Cab. azorensis* MV1087^T were obtained from Wery *et al.* (2001) and Hania *et al.*(2015a). +; positive result, -; negative result, ND; not determined.

Characteristics	1	2	3	4	5	6
Cell Morphology						
Cell width	0.7	0.5	0.3–0.6	0.5	0.3–0.5	0.4–0.6
Cell length	5.0–23.0	5.0–10.0	2.0–6.8	3.0–8.0	0.5–2.0	5.0–10.0
Gram stain	-	+	-	+	-	+
Spore formation	+	+	-	+	ND	+
Yeast extract dependence	+	+	-	-	+	+
Growth range (optimum)						
Temperature (°C)	15–40 (29)	20–48 (40)	16–44 (37)	20–50 (42)	45–65 (45)	40–65 (55)
NaCl (%, w/v)	1–5 (2)	0.5–15 (4)	0–8 (1.5)	1–10 (5)	0.65–6.5(2)	2–15 (5)
pH	5.0–9.0 (6.5)	6.2–8.1 (6.9)	6.0–9.0 (7.5)	5.5–8.5 (6.8)	5.5–9.0 (7.0)	5.0–9.0 (7.0)
DNA G+C content (mol%)	28.8	30.7	28.2	33.0	27.0	30.7
Reduction of sulfur compounds					Sulfur	Thiosulfate
Substrates used						
Casamino acids	-	-	+	+	+	-
Arginine, Starch, Xylose, Mannitol	-	-	-	-	+	-
Cystein, Serine, Lysine, Succinate	-	-	-	+	-	-
Valine	-	-	+	+	-	-
Glutamic acid	-	-	+	-	-	-
Methionine	+	-	+	-	-	-
Glycine	-	-	+	-	-	-
Fumarate	-	-	-	+	-	-
Fructose	+	+	+	-	+	+
Glucose	-	+	+	-	+	+
Galactose	-	-	-	-	+	(+)
Mannose	+	-	-	-	+	+
Ribose	-	-	+	-	+	-
Glycerol	-	-	ND	-	-	-
Cellobiose	+	-	-	-	-	+
Mannitol	-	+	-	-	+	-
Acetate	-	+	-	-	-	-

elemental sulfur, sulfate and thiosulfate were not reduced by the strain. The nitrate/nitrite reduction assay showed nitrogen compounds were not reduced by strain MCWD3^T. Fructose, cellobiose, mannose, and methionine stimulated growth in the presence of yeast extract, but none of the other organic acids, amino acids or carbohydrates tested were stimulatory, which are different characteristics from other closely related species including *S. faouarense* SOL3f3^T (Table 1). The HPLC assay confirmed acetate was a fermentation product from the yeast extract, and other organic acids such as formate, propionate, and butyrate were not detected.

These physiological properties distinguished strain MCWD3^T from other members of this clade. It stains Gram negative while *C. paucivorans* 37HS60^T, *S. faouarense* SOL3f37^T and *Csb. kiririmariensis* L21-TH-D2, stain Gram positive. It is mesophilic and fails to grow at temperatures > 40°C, distinguishing it from *Cab. azorensis* MV1087^T (Wery *et al.*, 2001) and *Csb. kiririmariensis* L21-TH-D2^T (Hania *et al.*, 2015a). Unlike *S. faouarense* SOL3f37^T (Rezgui *et al.*, 2011) and *Csb. kiririmariensis* L21-TH-D2^T, strain MCWD3^T does not tolerate high concentrations of NaCl. In addition, the pattern of substrates utilized by strain MCWD3^T differs from that of all the other species.

Chemotaxonomy

According to the draft genome sequence of strain MCWD3^T (LOHE00000000.1), the DNA G+C content is 28.8 mol%.

The predominant fatty acid components (> 5%) are iso-$C_{13:0}$ (5.4%), iso-$C_{15:0}$ (30%), antesio-$C_{15:0}$ (7.6%), $C_{15:0}$ (5.0%), iso-$C_{15:0}$ DMA (16.9%), summed feature 5 ($C_{15:0}$ DMA and/or $C_{14:0}$ 3OH, 9.7%), and $C_{16:0}$ DMA (5.2%) (Table 2). The dominance of the iso-$C_{15:0}$ and iso-$C_{15:0}$ DMA are also reported for other genera in the clade. The polar lipid profiles of strain MCWD3^T included diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and an unidentified lipid, two unidentified phospholipids (PL) and aminolipids (AL), and five unidentified aminophospholipids (APL) (Supplementary data Fig. S1). In the *Cts. paucivorans* 37HS60^T (Liebgott *et al.*, 2008), PG and PE were detected, but DPG was not. The presence of DPG, PG and PE is also reported for *S. faouarense* SOL3F37^T (Rezgui *et al.*, 2011) and *B. mesophilus* BM^T (Fang *et al.*, 2012), but amount of DPG appears to be higher than the PG and PE, which is different from MCWD3^T.

Taxonomic conclusion

The morphological, phylogenetic, biochemical, and chemo-

Table 2. Cellular fatty acid composition (%) of strain MCWD3^T and phylogenetically closely related species. Strains 1. MCWD3^T, 2. *Sporosalibacterium faouarense* SOL3f37^T, 3. *Brassicibacter mesophilus* BM^T, 4. *Clostridiisalibacter paucivorans* 37HS60^T, 5. *Caloranaerobacter azorensis* MV1087^T, 6. *Caldisalinibacter kiririmariensis* L21-TH-D2^T. Fatty acids patterns of strain MCWD3^T and *Sporosalibacterium faouarense* SOL3f37^T are obtained from this study and all others are from Hanai *et al.* (2015b). Fatty acids lower than 1% in all strains were not reported.

Fatty acids	1	2	3	4	5	6
Saturated						
$C_{14:0}$	2.6	3.7	6.1	16.1	1.0	1.0
$C_{15:0}$	5.0					
$C_{16:0}$	1.6	2.1	1.1	2.7	0.8	4.2
$C_{18:0}$			0.6	1.1	-	4.2
Saturated & branched						
iso-$C_{13:0}$		6.0	6.7	1.5	0.7	0.7
iso-$C_{14:0}$				1.4		
iso-$C_{15:0}$	30.0	47.3	41.1	20.4	57.5	49.5
anteiso-$C_{15:0}$	7.6	5.2	3.1	4.3	3.7	3.5
iso-$C_{17:0}$					1.5	3.2
Acetal/Aldehyde form						
$C_{14:0}$ DMA	3.9	2.4	2.8	7.4		
$C_{16:0}$ ALDE		1.2		4.7		2.4
$C_{16:0}$ DMA	5.3	1.9	1.6	9.4	1.0	
$C_{18:0}$ DMA			0.8	2.4		0.8
iso-$C_{15:0}$ ALDE	4.4	10.3	7.2	3.1	3.9	4.9
iso-$C_{15:0}$ DMA	16.9	12.9	20.3	5.2	21.4	15.3
ante-$C_{15:0}$ DMA	2.7		2.1	0.6	1.3	1.0
Unsaturated						
$C_{16:1}$ cis9			0.8	1.2		
$C_{16:1}$ cis9 DMA			0.9	3.8		
$C_{17:1}$ cis11		1.6				
Others						
Summed feature 1*	-	2.4	1.2	4.7		
Summed feature 4 *	4.9			3.1		
Summed feature 5 *	9.8					
$C_{17:0}$ cyclo				2.2		
ECL 17.103				1.4	4.4	2.5

ALDE: aldehyde, DMA: dimethyl acetal, cyclo: cyclopropyl fatty acid, C: *cis* isomer, iso: iso-branched fatty acid, ante: anteiso-branched fatty acid
Summed feature 1 =$C_{13:1}$ cis12 and/or $C_{11:1}$ 2OH, Summed feature 4 =$C_{15:2}$ and/or $C_{15:1}$ cis7, Summed feature 5 =$C_{15:0}$ DMA and/or $C_{14:0}$ 3OH

taxonomic analyses indicate that strain MCWD3^T is distinct from members of other genera in the family *Clostridiaceae*. Thus, we propose that strain MCWD3^T is a novel species in a new genus with the name *Abyssisolibacter fermentans*.

Description of *Abyssisolibacter* gen. nov.

Abyssisolibacter (A.bys'si.so.li.bac'ter. L. gen. n. *Abyss* deep, L. n. *solum* soil, N.L. masc. n. *bacter* a rod, *Abyssisolibacter* deep-sea sediment (dwelling) bacteria).

Cells are anaerobic, Gram-stain-negative, mesophilic, thin rod-shaped. Yeast extract and NaCl are required for growth. Ferment a few carbohydrates and amino acids. The major cellular fatty acids are iso-$C_{15:0}$ and iso-$C_{15:0}$ DMA and polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and several unidentified species. The DNA G+C content is approx. 29 mol%. Phylogenetic analysis suggests that this genus belongs to the family *Clostridiaceae*. The type species is *Abyssisolibacter fermentans*.

Description of *Abyssisolibacter fermentans* sp. nov. *fermentans* (fer.men'tans. L. part. adj. *fermentans* fermenting)

Cells are long thin rods, size of 0.7 × 5.0–23.0 μm. Colonies were bright yellow, circular and smooth. The temperature for growth is 15–40°C (optimum 29°C), and the pH range is 6.0–9.0 (optimum pH 6.5). Requires 1–5% (w/v) NaCl for growth, with an optimum at 2.0%. The fermentation product from yeast extract broth is acetate. It is heterotrophic, CO_2 was not used as carbon source. Cells are not capable of reducing elemental sulfur, sulfate and thiosulfate. Cells metabolize fructose, mannose, cellobiose and methionine in the presence of yeast extract (0.5 g/L). The major fatty acids identified were $C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$ DMA, iso-$C_{15:0}$ DMA, anteiso-$C_{15:0}$ DMA, Summed feature 4 (comprised with $C_{15:2}$ and/or $C_{15:1}$ cis7) and Summed feature 5 ($C_{15:0}$ DMA and/or $C_{14:0}$ 3OH). In addition to the genus description, two unknown phospholipids, two unknown aminolipids, and five unknown aminophospholipids were present. The type strain, MCWD3^T (= KCTC 15524^T), was isolated from Ulleung Basin sediment in the East Sea, Korea.

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