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Sphingopyxis granuli sp. nov., a β-Glucosidase-Producing Bacterium in the Family *Sphingomonadaceae* in α-4 Subclass of the *Proteobacteria*

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Strain Kw07^T, a Gram-negative, non-spore-forming, rod-shaped bacterium, was isolated from granules in an Up-flow Anaerobic Sludge Blanket (UASB) bioreactor used in the treatment of brewery wastewater. 16S rRNA gene sequence analysis revealed that strain Kw07^T belongs to the α-4 subclass of the *Proteobacteria*, and the highest degree of sequence similarity was determined to be to *Sphingopyxis macrogoltabida* IFO 15033^T (97.8%). Chemotaxonomic data revealed that strain Kw07^T possesses a quinone system with the predominant compound Q-10, the predominant fatty acid $C_{18:1}$ ω7*c*, and sphingolipids, all of which corroborated our assignment of the strain to the *Sphingopyxis* genus. The results of DNA-DNA hybridization and physiological and biochemical tests clearly demonstrated that strain Kw07^T represents a distinct species. Based on these data, Kw07^T (= KCTC 12209^T = NBRC 100800^T) should be classified as the type strain for a novel *Sphingopyxis* species, for which the name *Sphingopyxis granuli* sp. nov. has been proposed.

Key words: *α-Proteobacteria, Sphingomonadaceae, Sphingopyxis granuli*

The genus *Sphingomonas* was created by Yabuuchi *et al.* (1990), in order to designate Gram-negative, strictly aerobic, chemoheterotrophic, yellow-pigmented, and rod-shaped bacteria, which harbor glycosphingolipids as cell envelope components. The genus contained a multitude of species consisting of strains of clinical origin, as well as from a variety of other environments. The relatively diverse species of the *Sphingomonas* genus have been classified into at least four clusters within the α-4 subclass of the *Proteobacteria*. The *Sphingomonas* genus has also been divided into four genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* (Takeuchi *et al.*, 2001).

Yabuuchi *et al.* (2002) suggested that the genus *Sphingomonas* should remain undivided, and that the species, *Novosphingobium*, *Sphingobium*, and *Sphingopyxis* constitute objective synonyms of species of the *Sphingomonas* genus. After that, however, Busse *et al.* (2003) suggested that sym-homospermidine, which is a characteristic marker within the *Sphingomonadaceae* family, had not been considered in the taxonomic considerations of Yabuuchi *et al.* (1990, 2002), and strongly advocated Takeuchi's proposal. Presently, a great many articles have currently adopted the nomenclature of Takeuchi *et al.* (2001) in their reports of new species in the *Novosphingobium* (Fujii *et al.*, 2003; Sohn *et al.*, 2004; Tiirola *et al.*, 2004), *Sphingobium* (Ushiba *et al.*, 2003), *Sphingomonas* (Busse *et al.*, 2003; Li *et al.*, 2004; Rivas *et al.*, 2004) and *Sphingopyxis* genera (Kämpfer *et al.*, 2002; Godoy *et al.*, 2003; Yoon and Oh, 2005).

The genus *Sphingopyxis* currently contains six validated species, *Sphingopyxis alaskensis* (Vancanneyt *et al.*, 2001; Godoy *et al.*, 2003), *S. chilensis* (Godoy *et al.*, 2003), *S. flavimaris* (Yoon and Oh, 2005), *S. macrogoltabida* (Takeuchi *et al.*, 1993, 2001), *S. terrae* (Takeuchi *et al.*, 1993, 2001), and *S. witflariensis* (Kämpfer *et al.*, 2002).

Anaerobic granules are bacterial aggregates originating from the flocculation of bacterial sludge in Up-flow Anaerobic Sludge Blanket (UASB) reactors (de Zeeuw and Lettinga, 1980). They are composed of microorganisms, inorganic nuclei, and extracellular polymers (Fukuzaki *et al.*, 1991; Shen *et al.*, 1993). A great deal of attention has been paid to the internal structure and catalytic activities exhibited by these granules (MacLeod *et al.*, 1990; Schmidt and Ahring, 1996). In a series of studies, we attempted to isolate microorganisms from these anaerobic granules, in order to characterize the community structure, using a culture system. Interestingly, the granules were determined to harbor aerobic bacteria,

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although they had been maintained in anaerobic conditions for a period of two years. Strain Kw07^T is one of the aerobic bacterial isolates obtained from the granules. In this study, we used a polyphasic approach, which included phylogenetic analysis predicated on 16S rRNA gene sequences, as well as chemotaxonomic and phenotypic properties, in order to determine the precise taxonomic position of strain Kw07^T. The results obtained in this study demonstrate that strain Kw07^T can be considered to be a member of the genus *Sphingopyxis,* using the nomenclature provided by Takeuchi *et al.* (2001), but that it can also be clearly distinguished from the *Sphingopyxis* species with other validly published names. Here, we propose that strain Kw07^T should appropriately be considered the type strain of a novel species, which we have named *Sphingopyxis granuli* sp. nov.

Materials and Methods

Isolation of bacterial strain and culture condition

Strain Kw07^T was isolated from granules via direct plating onto R2A agar (Difco, USA). In order to isolate the strain, we homogenized brownish black granules (around 2 mm in diameter) from a brewery wastewater-treating UASB reactor, using an Ace Homogenizer (Nihonseiki, Japan). The suspension was then spread onto R2A agar plates after serial dilution with 50 mM phosphate buffer (pH 7.0). The plates were then incubated at 30°C for two weeks. Single colonies on these plates were purified by transferring them onto new plates, and subjecting them to an additional incubation under the same conditions as before. The purified colonies were tentatively identified by using the partial 16S rRNA gene sequences. Strain Kw07^T was one of isolates which appeared predominantly on the plates under aerobic conditions. This organism was then submitted to the Korean Collection for Type Cultures (= KCTC 12209^T) and the National Institute of Technology and Evaluation (= NBRC 100800^T) in Japan.

Morphological and physiological characterization

Cell morphology and motility were observed with a Nikon light microscope (1,000 × magnification), with the cells being allowed to grow for 3 days at 30°C, on R2A agar. Gram reactions were conducted according to the non-staining method, as was described by Buck (1982). Oxidase activity was evaluated via the oxidation of 1% *p*-aminodimethylaniline oxalate. Catalase activity was determined by measurements of bubble production after the application of 3% (v/v) hydrogen peroxide solution. Acid production from carbohydrates was assessed by the procedures outlined in "Microbiology; a Laboratory Manual" (Cappuccino and Sherman, 2002). Growth at a variety of temperatures (4, 15, 25, 30, 37 and 42°C) was assessed on R2A agar, and growth at a variety of pH values (4, 5, 6, 7, 8, 9 and 10) was assessed on R2A broth. Growth on Nutrient agar, trypticase soy agar (TSA), and MacConkey agar was also evaluated, at 30°C. The API 20NE, API ID32 GN, and API ZYM microtest systems were employed in these tests, according to the recommendations of the manufacturer (bioMérieux, France).

Chemotaxonomic characterization

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and then purified via TLC (thin-layer chromatography) on Merck Kieselgel 60 F_{254} plates (20 × 20 cm, 0.5 cm thick), using a mixture of petroleum benzene and diethyl ether (85:15, v/v) as the solvent. The extracts were subsequently analyzed by HPLC, as previously described (Collins and Jones, 1981; Shin *et al.*, 1996). In order to perform fatty acid methyl ester analysis, the strains were allowed to grow on TSA for 48 h at 30°C, and then two loops of the well-grown cells were harvested. Fatty acid methyl esters were prepared, separated, and identified with the Sherlock Microbial Identification System (MIS), produced by MIDI, USA (Sasser, 1990).

The total cellular lipids were extracted three times from 50 mg of dried cells, using 5 ml of chloroform/methanol (2:1, v/v). A portion of these extracted lipids was then subjected to mild alkaline hydrolysis with 0.5 M KOH in chloroform and methanol (2:1, v/v) for 1 h at 40°C, as was described by Yabuchi *et al.* (1990). Both the total extracted lipids, and their alkaline hydrolysates, were then analyzed via TLC (HPTLC plate, Silica gel 60, 10 x 10 cm; Merck, USA) with a solvent system consisting of chloroform, methanol, and water (70:30:5, v/v). In order to discern the glycolipid spots, we sprayed orcine-sulfuric acid reagent (0.2% orcinol in 2 M sulfuric acid) and charred the samples at 120°C until maximum purple color had developed.

Determination of G + C content

The genomic DNA of strain Kw07^T was extracted and purified with the QIAGEN Genomic-tip system 100/G (QIAGEN, Japan), and was then enzymatically degraded into nucleosides, as described previously (Tamaoka and Komagata, 1984; Mesbah *et al.*, 1989). A 10 μl volume of a solution which contained 10 μg of DNA was heated in a boiling water bath for 5 min, then rapidly cooled in an ice-water bath. The denatured DNA solution was mixed with 10 μl of nuclease P1 solution (20 U/ml), and incubated at 37°C for 1 h. 10 μl of glycine buffer (pH 10.0) and 10 μl of alkaline phosphatase (10 U/ml) were added to the sample, and incubated for 3 h at 37°C. The obtained nucleoside mixture was then separated by HPLC, using a Waters Nova-Pak C_{18} column (3.9 × 300 mm), and was eluted by a mixture of 0.2 M $(NH_4)H_2PO_4$ and acetonitrile (40:1, v/v) at a flow rate of 0.7 ml/min, and detected by its UV absorbance at 270 nm. *E. coli* DNA (Sigma, USA) was used as the calibration reference.

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was extracted and purified with the Genomic DNA Isolation Kit (Core Bio System, Korea). The 16S rRNA gene was amplified from the chromosomal DNA of strain Kw07^T using the universal bacterial primer set, 9F [5'-GAGTTTGATCCTGGCTCAG-3'; positions 9-27 (*Escherichia coli* 16S rRNA numbering)] and 1512R [5'-ACGGTTACCTTGTTACGACTT-3'; positions 1512-1492] (Weisburg *et al.*, 1991). PCR amplification was conducted in a 100 μl final reaction volume, and the reaction mixture consisted of each primer at a concentration of 1 μM, 100 ng of extracted DNA, each deoxynucleoside triphosphate at a concentration of 0.1 μM, 10X reaction buffer, and 2.5 U of *Taq* DNA polymerase. PCR was conducted for 35 cycles, with the following thermal profile: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 2 min. The final cycle included a 10 min extension step at 72°C. The PCR products were purified with an *AccuPrep*™ PCR purification kit (Bioneer, Korea), according to the instructions of the manufacturer. The purified PCR products were sequenced by Genotec, Korea. The primers used for full sequencing were as follows: 9F, 1512 R, 341F [5'-CCTA-CGGGAG-GCAGCAG-3'; positions 341-357 (*E. coli* 16S rRNA numbering)], 907F [5'-AAACTCA-AAKGAATTGACGG-3'; positions 907-926], 536R [5'-GTATTACCGCGGCT-GCTG-3'; positions 536-519] and 1100R [5'-GGGTTG-CGCTCGTTG-3'; positions 1114-1110]. The full sequence of the 16S rRNA gene was compiled with SeqMan software, and the 16S rRNA gene sequences of the test strain were edited using the BioEdit program (Hall, 1999). The 16S rRNA gene sequences of the related taxa were obtained from GenBank. Multiple alignments were performed with the CLUSTAL X program (Thompson *et al.*, 1997). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed via the neighbor-joining method (Saitou and Nei, 1987) in the MEGA 2 Program (Kumar *et al.*, 2001). Bootstrap analysis with 1,000 replicates was also conducted, in order to obtain confidence levels for the branches (Felsenstein, 1985). All of the species in the *Sphingopyxis* genus were included in the phylogenetic tree, and *Sphingomonas taejonensis* was also included, as it appeared to be closely phylogenetically related.

DNA-DNA hybridization

DNA-DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was conducted in five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the remaining three values were utilized in the calculation of similarity values. The DNA relatedness values quoted are expressed as the means of these three values.

Results and Discussion

Cultural and physiological characteristics

Strain Kw07^T was cultured on R2A agar at 30°C, yielding a white-colored, circular, and non-glossy appearance on that agar. Strain Kw07^T was found to be an aerobic, Gram-negative, motile, and rod-shaped bacterium. Strain Kw07^T was also determined to be able to grow at 25-37°C, but did not grow at 4 and 42°C. Growth at 30°C was also observed on nutrient agar and trypticase soy agar (TSA). Results regarding the physiological characteristics of strain Kw07^T are summarized in the species description, and a comparison of selective characteristics with related type strains is shown in Table 1. Strains of the genus *Sphingopyxis* are all Gram-negative, rod-shaped bacteria. All strains exhibit both oxidase and catalase activity. All strains test negative for the reduction of nitrates to N_2, indole production, and the production of acid from glucose. All strains also test negative for arginine dihydrolase, protease (gelatin hydrolysis), and urease activity. All strains test positive for the assimilation of 3-hydroxybutyrate, L-proline, and suberate. All strains are

Table 1. Differential phenotypic characteristics between strain Kw07^T and related taxa

	1	2	3	4	5	6
Nitrate reduction to NO_2	+	-	-	-	-	-
Enzyme activity						
β-galactosidase	-	-	-	-	+	+
β-glucosidase (esculin hydrolysis)	+	+	+	-	+	+
Assimilation test						
Acetate	+	-	-	-	-	+
Adipate	-	-	+	-	-	-
D-Glucose	-	+	+	+	+	+
D-Mannose	-	-	+	-	-	-
L-Alanine	+	+	-	-	+	+
L-Histidine	+	-	+	+	-	+
L-Serine	-	+	-	-	-	-
Malate	-	+	+	-	+	-
Maltose	-	+	+	+	-	-
N-Acetyl-glucosamine	-	-	+	-	-	-
Propionate	+	-	-	-	+	+
Salicin	+	-	-	-	-	-
Valerate	+	-	-	-	+	+

Taxa: 1, Kw07^T; 2, *Sphingopyxis alaskensis* DSM 13593^T; 3, *Sphingopyxis chilensis* DSM 14889^T; 4, *Sphingopyxis macrogoltabida* DSM 8826^T; 5, *Sphingomonas taejonensis* KCTC 2884^T; 6, *Sphingopyxis witflariensis* DSM 14551^T.

All the strains were grown and compared together. +, Positive; -, negative.

Table 2. Cellular fatty acid profiles of strain Kw07^T and related taxa

Compound	1	2	3	4	5	6
Saturated fatty acids	-	-	-		-	-
$C_{12:0}$	-	-	-		-	-
$C_{14:0}$	1.1	-	1.1	-	1.5	-
$C_{15:0}$		3.6	-		1.3	5.2
$C_{16:0}$	13.6	6.9	7.2	14.1	21.6	8.8
$C_{17:0}$		3.4			1.6	2.5
$C_{18:0}$	1.0	1.1	1.0	1.1	5.5	1.4
$C_{19:0}\Delta\omega 8c$					1.1	-
Unsaturated fatty acids						-
$C_{16:1}\omega 5c$	2.7	1.0	1.1	2.7	-	1.5
$C_{17:1}\omega 6c$	2.6	39.1	3.6		3.3	34.1
$C_{17:1}\omega 8c$		8.5	-			5.7
$C_{18:1}\omega 5c$	1.8				4.1	-
$C_{18:1}\omega 7c$	38.6	14.3	30.6	35.8	26.7	11.2
$C_{18:1}\omega 9c$	0.6		-		3.3	1.0
Hydroxy fatty acids						-
$C_{14:0}$2OH	4.1	1.3	10.3	2.8		1.2
$C_{15:0}$2OH		8.2	1.9		-	7.6
$C_{16:0}$2OH	2.5	1.5	3.6	2.8	2.5	1.6
$C_{16:0}$iso 3OH			1.4			-
$C_{17:0}$iso 3OH					1.3	-
Summed feature						-
SF 4	29.6	6.8	34.2	38.4	17.4	16.3
SF 6	1.0	-	1.0	1.5	2.7	-

Taxa: 1, Kw07^T; 2, *Sphingopyxis alaskensis* DSM 13593^T; 3, *Sphingopyxis chilensis* DSM 14889^T; 4, *Sphingopyxis macrogoltabida* DSM 8826^T; 5, *Sphingomonas taejonensis* KCTC 2884^T; 6, *Sphingopyxis witflariensis* DSM 14551^T. All strains were grown and compared together. Fatty acids that account for less than 1% of total fatty acids are shown as - . For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. Summed features designate groups of two or three fatty acids which cannot be separated by GLC with the MIDI system. Summed Feature 4 contained one or more of the fatty acids $C_{16:1}$ ω7*c* and $C_{15:1}$ iso 2-OH; Summed Feature 6 contained $C_{18:2}$ ω6,9*c* and $C_{18:0}$ anteiso.

negative for the assimilation of 2-ketogluconate, 3-hydroxybenzoate, 4-hydroxybenzoate, 5-ketogluconate, caprate, citrate, DL-lactate, D-melibiose, D-ribose, D-sorbitol, D-sucrose, gluconate, glycogen, itaconate, L-arabinose, L-fucose, L-rhamnose, malonate, mannitol, myo-inositol, and phenyl acetate.

Chemotaxonomic characteristics

The cellular fatty acid profiles of strain Kw07^T and the related *Sphingopyxis* reference strains are shown in Table 2. The major cellular fatty acids in strain Kw07^T included: octadecanoic acid ($C_{18:1}$ ω7*c*, 38.6%), summed feature 4 ($C_{16:1}$ ω7*c* / $C_{15:0}$ iso 2-OH, 29.6%) and hexadecanoic acid ($C_{16:0}$, 13.6%). Minor amounts of the 2-hydroxy fatty acids, $C_{14:0}$ 2-OH (4.1%) and $C_{16:0}$ 2-OH (2.5%), were also determined to be present. The presence of 2-OH fatty acids (2-OH myristic acid and 2-OH palmitic acid), rather than 3-OH fatty acids, octadecanoic acid, and summed feature 4, as major fatty acids in strain Kw07^T were characteristic features of members of the genus *Sphingopyxis,* as has been previously discussed (Godoy *et al.*, 2003). Significant differences in fatty acid profiles were found in the other *Sphingopyxis* species, *S. alaskensis* and *S. witflariensis,* both of which presented $C_{17:1}$ ω6*c* as their predominant fatty acid.

Q-10 was the predominant ubiquinone of strain Kw07^T. The quinone system supported our assignment of strain Kw07^T to the *α-Proteobacteria,* in which the majority of species (including *Sphingopyxis* species) also exhibit Q-10 as the predominant quinone (Collins and Jones, 1981).

Strain Kw07^T was also determined to harbor sphingolipids.

Phylogenetic analysis

The 16S rRNA gene sequence of strain Kw07^T was found to be a continuous stretch of 1422 (positions 19-1501; *Escherichia coli* numbering) nucleotides. The 16S rRNA gene sequences of the related taxa were obtained from GenBank. Strain Kw07^T was determined to belong to the alpha-4 subclass of the *Proteobacteria,* and the highest degrees of sequence similarity were found to be with *Sphingopyxis macrogoltabida* DSM 8826^T (97.8%), *Sphingopyxis witflariensis* DSM 14551^T (97.6%), and *Sphingopyxis chilensis* DSM 14889^T (97.4%). The extent of sequence similarity shared between strain Kw07^T and any validly described species in a related genus was less than 93.1% (*Porphyrobacter neustonensis* DSM 9434^T). In the phylogenetic tree (Fig. 1), strain Kw07^T clearly belonged to the *Sphingopyxis* lineage, as evidenced by the high bootstrap value.

G + C content and DNA-DNA hybridization

The G+C content of the genomic DNA of strain Kw07^T was 63.7%. Strain Kw07^T exhibited a relatively low level of DNA-DNA similarity to the type strains, *Sphingopyxis macrogoltabida* DSM 8826^T (25.0%), *S. witflariensis* DSM 14551^T (30.4%), *S. chilensis* DSM 14889^T (20.2%), *S. alaskensis* DSM 13593^T (19.6%), and *Sphingomonas taejonensis* KCTC 2884^T (11.9%). The DNA-DNA hybridization level was determined to be less than 70% (Stackebrandt and Goebel, 1994), which is the threshold which delineates a genomic species. Our results, then, support the designation of strain Kw07^T as a separate, previously unrecognized species.

The results of polyphasic analysis supported the recognition of a novel species within the genus *Sphingopyxis,* for which the name *Sphingopyxis granuli* sp. nov. has been proposed.

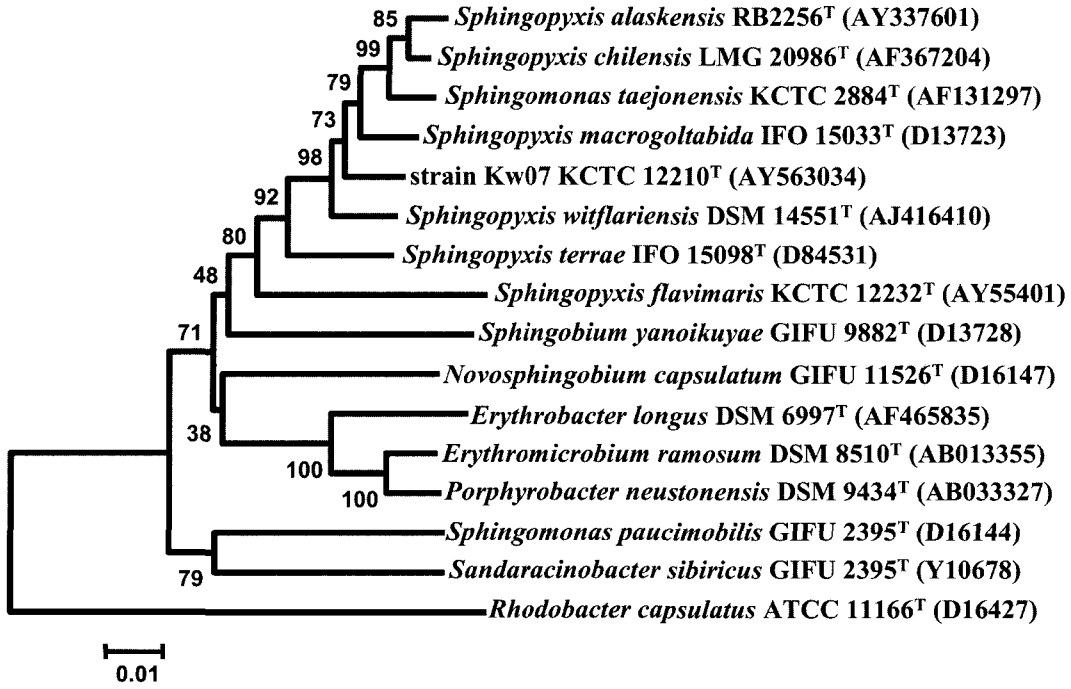

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationships between *Sphingopyxis* species and related genera (neighbor-joining method). A bar represents 0.01 substitutions per nucleotide position.

Description of Sphingopyxis granuli sp. nov.

Sphingopyxis granuli (L. gen. n. granuli, of a small grain pertaining to the granule, from which the type strain was isolated). Cells are Gram-negative, motile, non-spore-forming rods (approx. 1 μm in length). They are oxidase-positive, exhibiting oxidative metabolism (obligately aerobic), and are catalase positive. Favorable growth occurs aerobically on R2A agar, nutrient agar, and TSA, at 30 °C; optimally at pH 6.0-8.5; forms white and semi-translucent, but not shiny, colonies with whole edges within 3 days, with diameters of approximately 1.5 -3 mm. Q-10 is the predominant quinone . The primary cellular fatty acids include octadecanoic acid ($C_{18:1}$ ω7*c*, 38.6%), summed feature 4 ($C_{16:1}$ ω7*c* / $C_{15:0}$ iso 2-OH, 29.6%), and hexadecanoic acid ($C_{16:0}$, 13.6%). Minor quantities of the 2-hydroxy fatty acids $C_{14:0}$ 2-OH (4.1%) and $C_{16:0}$ 2-OH (2.5%) are also manifest, and constitute a characteristic of members of the genus *Sphingopyxis*. Sphingolipids are also present. The genomic G + C content of the genomic DNA is 63.7 mol%. Carbon and nitrogen source utilization and enzymatic activity are shown in Table 1.

The type strain, Kw07^T (= KCTC 12209^T) was isolated from granules obtained from the wastewater treatment plant of a brewery in Kwang-Ju, Republic of Korea.

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