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Flavihumibacter profundi sp. nov., isolated from eutrophic freshwater sediment[§]

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A Gram-stain-positive, aerobic, non-motile, non-spore-forming, and rod-shaped bacterium, designated strain CHu64-6-1^T, was isolated from a 67-cm-long sediment core collected from the Daechung Reservoir at a water depth of 17-m in Daejeon, Republic of Korea. Comparative 16S rRNA gene sequence studies placed the new isolate in the class *Sphingobacteriia*, and the isolate is notably most closely related to *Flavihumibacter sediminis* CJ663^T (98.1% similarity), *Flavihumibacter solisilvae* 3-3^T (97.8%), *Flavihumibacter petaseus* T41^T (97.5%), *Flavihumibacter cheonanensis* WS16^T (97.4%), and *Flavihumibacter stibioxidans* YS-17^T (97.2%). The cells of strain CHu64-6-1^T formed yellow colonies on R2A agar and contained MK-7 as the only menaquinone, phosphatidylethanolamine, an unidentified phospholipid, and two unidentified aminolipids as the major polar lipids, and $C_{15:0}$ iso, $C_{17:0}$ iso 3-OH, $C_{15:1}$ iso G, and $C_{16:1}$ *ω5c* as the major fatty acids (> 5%). The DNA G + C content of the genome was determined to be 46.5 mol%. The DNA-DNA hybridization values of strain CHu64-6-1^T with *F. sediminis* CJ663^T, *F. solisilvae* 3-3^T, *F. petaseus* T41^T, *F. cheonanensis* WS16^T, and *F. stibioxidans* YS-17^T were 12.4–33.2%. Based on the combined genotypic and phenotypic data, we propose that strain CHu64-6-1^T represents a novel species of the genus *Flavihumibacter*, for which the name *Flavihumibacter profundi* sp. nov. is proposed. The type strain is CHu64-6-1^T (= KCTC 62290^T = CCTCC AB 2018060^T).

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain CHu64-6-1^T is MF770247.

Keywords: *Flavihumibacter*, *Flavihumibacter profundi*, CHu64-6-1, sediments

Introduction

Zhang *et al.* (2010) established the genus *Flavihumibacter*, based on the single species *Flavihumibacter petaseus* within the family *Chitinophagaceae*. Five species have been validly described, *F. cheonanensis* (Kim *et al.*, 2014), *F. petaseus*, *F. sediminis* (Lee and Cha, 2016), *F. solisilvae* (Lee *et al.*, 2014), and *F. stibioxidans* (Han *et al.*, 2016). All the members of this genus were isolated from soil or sediment producing flexirubin-type pigments and are Gram-stain-positive, non-motile, aerobic and rod-shaped, and positive for oxidase but negative for nitrate reduction. We recovered a novel strain, designated CHu64-6-1^T, during the course of a study on iron and sulphur oxidizing microbial diversity in the sediment of the Daechung Reservoir (Jin *et al.*, 2017). Here, we establish the taxonomic position of strain CHu64-6-1^T, which should represent a new species within the genus *Flavihumibacter* based on the 16S rRNA gene sequence analysis, using a polyphasic approach.

Materials and Methods

Isolation and culture condition

A bacterial strain, designated CHu64-6-1^T, was isolated using diluted R2A agar (Difco) (per L: 0.05 g peptone, 0.05 g yeast extract, 0.05 g casamino acid, 0.05 g dextrose, 0.05 g soluble starch, 0.03 g K_2HPO_4, 0.005 g $MgSO_4$, 0.03 g sodium pyruvate, and 15 g agar) from a 67-cm-long sediment core collected from the Daechung Reservoir at a water depth of 17 m in Daejeon, South Korea. The strain CHu64-6-1^T was recovered using several dilutions to extinction (10^{-6} or 10^{-7}) in R2A medium. A 100 μl sub-sample of the suspended material was aseptically spread onto modified 1/10 R2A agar and incubated at 25°C under heterotrophic conditions. A yellow-pigmented bacterial strain was isolated after 6 days and routinely subcultivated on R2A agar at 30°C for 48 h and kept in a glycerol solution (20%, v/v) at -70°C for long-term preservation. For the next experiments, all strains were cultivated on R2A agar (Difco) at 30°C for 48 h. *F. cheonanensis* KACC 17467^T, *F. petaseus* KACC 17385^T, *F. sediminis* KACC 18874^T, *F. solisilvae* KACC 17917^T, and *F. stibioxidans* KCTC 52205^T were used as reference strains under the same conditions. The reference strains were purchased from the KACC (Korean Agricultural Culture Collection) and KCTC (Korean Collection for Type Cultures).

Morphological, physiological, and biochemical characterization

We used a Gram staining kit (Becton Dickinson) to carry out the Gram-staining experiment following the manufacturer's instructions. Cellular morphology and the mobility of strain CHu64-6-1^T was observed on a phase-contrast microscope (Nikon Optiphot, 1,000× magnification) using cells grown on R2A at 30°C for 24 h. Oxidase activity was assayed with 1% tetramethyl-*p*-phenylenediamine (Tarrand and Groschel, 1982) and catalase activity with 3% H_2O_2. The cell growth temperature (4, 8, 15, 20, 30, 37, and 42°C) was investigated on R2A agar. The pH range for cell growth from pH 5 to pH 10 at intervals of 1 unit was investigated in R2A broth, and different buffer systems for alkaline and acidic solutions were used as previously described (Jin *et al*., 2016). NaCl tolerance (0–5.0%, w/v) was determined on R2A agar for 7 days. API 20NE, ID 32 DN, and API ZYM kits (bioMérieux) and the Biolog GN2 MicroPlate were used to determine carbon-source utilization, enzyme activity and additional physiological characteristics according to the manufacturer's instructions. Duplicate antibiotic-susceptibility assays were performed using filter-paper disks containing the following: amikacin (30 μg/ml), ampicillin (10 μg/ml), amoxycillin (10 μg/ml), cefadroxil (30 μg/ml), cefoperazone (75 μg/ml), ceftazidime (30 μg/ml), ceftriaxone (30 μg/ml), chloramphenicol-C (30 μg/ml), ciprofloxacin (5 μg/ml), cloxacillin (1 μg/ml), co-trimoxazole (25 μg/ml), erythromycin (15 μg/ml), gentamicin (10 μg/ml), nalidixic acid (10 μg/ml), netillin (30 μg/ml), nitrofurantoin (300 μg/ml), norfloxacin (10 μg/ml), penicillin (10 units), tobramycin (10 μg/ml), and vancomycin (30 μg/ml). The discs were placed onto R2A plates that had been already spread with a culture of strain CHu64-6-1^T, and then, the plates were incubated at 30°C for 3 days. Susceptibility was considered positive for zones with diameters greater than 10 mm.

Chemotaxonomic characterization

For whole-cell fatty acid profiling, strains CHu64-6-1^T, *F. cheonanensis* KACC 17467^T, *F. petaseus* KACC 17385^T, *F. sediminis* KACC 18874^T, *F. solisilvae* KACC 17917^T, and *F. stibioxidans* KCTC 52205^T were cultured on TSA agar for 48 h, and the harvesting of bacterial cells was standardized by MIDI (http://www.microbialid. com/PDF/TechNote_101. pdf). To extract the fatty acids, we harvested the cell mass when the cells reached the late exponential phase. Separation and identification of the fatty acids were done by GC (Hewlett Packard 6890), and the TSBA 6 database provided the Sherlock software 6.1. Extraction of isoprenoid quinine was completed as described by Komagata and Suzuki (1987), and the analysis was done by HPLC (Shimadzu) with an YMC-Pack ODS-A column. The polar lipids were extracted and analyzed using two-dimensional TLC and identified following the method described by Tindall (1990). DNA G + C contents (mol%) were analyzed by HPLC after hydrolysis as described by Tamaoka and Komagata (1984).

Phylogenetic analysis

To extract the genomic DNA, we used the FastDNA^{TM} SPIN kit for soil DNA Extraction MP according to the manufacturer's instructions for Gram-negative bacteria. The purity of the DNA was checked on a ND1000 spectrometer (Nanodrop Technologies Inc.). To amplify the 16S rRNA gene, universal bacterial primer sets, 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′; *Escherichia coli* position 8-27) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′; *E. coli* position 1492-1510) (Lane, 1991), were used, and the conditions for the PCR cycling were as follows: 95°C for 5 min and 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min followed by a final extension step for 7 min at 72°C. Two more primers, 785F (5′-GGA TTA GAT ACC CTG GTA-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′), were used for the sequence analysis (Lane, 1991). The purified PCR products were then sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). To construct the phylogenetic trees, the sequence aligning software CLUSTAL X (Thompson *et al*., 1997), the editing software BIOEDIT (Hall, 1999) and MEGA 6 (Tamura *et al*., 2013) software were used. The close neighbor taxa of strain CHu64-6-1^T with validly published names were

Table 1. Phenotypic characteristics that differentiate strain CHu64-6-1^T from the most closely related species in the genus *Flavihumibacter*

Strains: 1, CHu64-6-1^T; 2, *F. cheonanensis* KACC 17467^T; 3, *F. petaseus* KACC 17385^T; 4, *F. sediminis* KACC 18874^T; 5, *F. solisilvae* KACC 17917^T; 6, *F. stibioxidans* KCTC 52205^T. All data are from this study, unless indicated. All strains were positive for aesculin hydrolysis, oxidase, *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase, but negative for nitrate reduction, indole production, glucose acidification, β-glucuronidase, or lipase (C14). None of them assimilated acetate, adipate, L-alanine, caprate, citrate, L-fucose, gluconate, L-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, 3-hydroxybutyrate, itaconate, 2-ketogluconate, 5-ketogluconate, DL-lactate, malate, malonate, D-mannitol, phenylacetate, propionate, D-ribose, L-serine, D-sorbitol, suberate, or valerate. +, positive; -, negative; w, weakly positive.

Characteristics	1	2	3	4	5	6
Catalase	-	-	+	+	+	+
Arginine dihydrolase	-	-	-	+	-	-
Urease	-	-	-	+	-	-
Gelatine hydrolysis	+	+	+	+	+	-
Carbon utilization :						
N-Acetyl-Glucosamine	-	+	+	+	+	+
L-Arabinose	-	+	+	+	+	+
D-Glucose	-	+	+	-	+	+
Glycogen	-	+	-	-	-	+
Inositol	-	+	-	-	-	-
Maltose	-	+	+	+	+	+
D-Melibiose	-	+	+	+	+	+
L-Proline	-	+	-	-	-	+
Rhamnose	-	+	-	-	-	+
Salicin	-	+	+	+	+	+
D-Sucrose	-	+	+	+	+	+
Enzyme activity :						
α-Chymotrypsin	-	-	-	+	-	w
α-Fucosidase	+	+	-	+	+	-
α-Mannosidase	+	-	-	-	-	w
DNA G + C content (mol%)	46.5	45.9[a]	48.1[b]	47.7[c]	49.5[d]	47.8[e]

[a] Data from Kim *et al.* (2014)
[b] Data from Zhang *et al.* (2010)
[c] Data from Lee and Cha (2016)
[d] Data from Lee *et al.* (2014)
[e] Data from Han *et al.* (2016)

Table 2. Cellular fatty acid compositions (%) of strain CHu64-6-1^T and the type strains of related species of the genus *Flavihumibacter*

Strains: 1, CHu64-6-1^T; 2, *F. cheonanensis* KACC 17467^T; 3, *F. petaseus* KACC 17385^T; 4, *F. sediminis* KACC 18874^T; 5, *F. solisilvae* KACC 17917^T; 6, *F. stibioxidans* KCTC 52205^T. Data are from the present study. tr, trace amount < 1%; -, not detected.

Fatty acids	1	2	3	4	5	6
$C_{13:0}$ iso	tr	tr	tr	tr	-	tr
$C_{13:0}$ anteiso	tr	-	-	tr	-	-
$C_{14:0}$ iso	tr	-	-	tr	1.5	-
$C_{14:0}$	tr	tr	tr	tr	-	tr
$C_{15:1}$ iso G	7.7	18.1	17.3	9.8	12.8	25.2
$C_{15:1}$ anteiso A	tr	1.1	tr	1.8	-	2.3
$C_{15:0}$ iso	34.0	41.0	33.0	31.5	33.3	32.3
$C_{15:0}$ anteiso	5.8	3.5	1.1	13.5	5.9	2.9
$C_{16:1}$ iso G	-	tr	tr	1.9	-	tr
$C_{16:0}$ iso	2.6	1.4	1.2	5.3	5.2	1.4
$C_{16:1}$ *ω5c*	7.3	3.8	6.2	2.2	5.6	6.4
$C_{16:0}$	3.4	2.8	2.7	3.1	3.4	2.1
$C_{15:0}$ iso 3 OH	4.1	3.3	4.1	2.4	6.1	4.2
$C_{15:0}$ 2 OH	tr	tr	tr	tr	-	-
$C_{15:0}$ 3 OH	tr	tr	1.1	-	2.4	tr
$C_{17:1}$ anteiso A	-	-	-	tr	-	-
$C_{17:0}$ iso	1.2	tr	tr	tr	-	tr
$C_{17:0}$ anteiso	1.2	-	-	tr	-	-
$C_{17:1}$ *ω6c*	1.3	-	tr	tr	-	-
$C_{17:0}$	tr	-	-	-	-	-
$C_{16:0}$ iso 3 OH	tr	tr	tr	2.0	-	tr
$C_{16:0}$ 2 OH	tr	-	-	-	-	-
$C_{16:0}$ 3 OH	1.0	1.5	2.7	1.5	3.7	1.3
$C_{17:0}$ iso 3 OH	14.9	18.2	16.2	14.5	16.0	16.0
$C_{17:0}$ 2 OH	5.0	tr	tr	4.6	-	tr
$C_{17:0}$ 3 OH	tr	1.3	1.9	tr	2.2	1.4
Summed Feature 3[a]	4.9	tr	8.7	1.4	1.9	0.8

[a] Summed feature 3 contains $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*.

identified with EzBioCloud (Yoon *et al*., 2017). The phylogenetic trees were completed using 3 different algorithms, neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Felsenstein, 1981) and maximum-likelihood (Fitch, 1971). Bootstrap values were calculated on 1,000 resamplings of the sequences (Felsenstein, 1985). The DNA-DNA relatedness of strain CHu64-6-1^T to the type strains *F. cheonanensis* KACC 17467^T, *F. petaseus* KACC 17385^T, *F. sediminis* KACC 18874^T, *F. solisilvae* KACC 17917^T, and *F. stibioxidans* KCTC 52205^T was performed with the fluorometric method described by Ezaki *et al*. (1989), and Salmon sperm DNA (Sigma; D7656) was used as a control.

Results and Discussion

Morphological, physiological, and biochemical characteristics

Visible colonies formed within 24 h on R2A agar when incubated at 30°C. Growth was found to occur at temperatures ranging from 15 to 37°C (weakly); however, no growth was observed at 8 and 42°C. Growth was found to occur at pH 5–8; however, no growth was observed at pH 4 or 9. The colonies were observed to be yellow, opaque, convex, smooth and circular with entire edges. The cells were found to be Gram-stain-positive, oxidase-positive but catalase-negative, non-motile, and rod-shaped. More detailed physiological and biochemical characteristics are listed in Tables 1 and 2 and in the species description.

Phylogenetic analysis

The almost-complete 16S rRNA gene sequence of strain

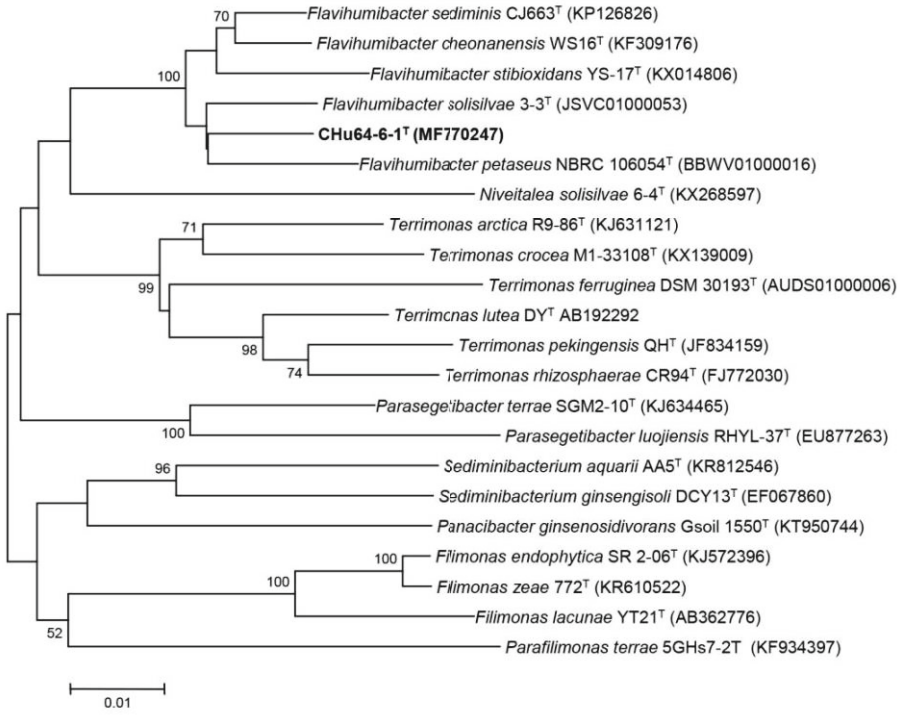

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain CHu64-6-1^T and related species. Numbers at branching points refer to bootstrap percentage (1,000 resamplings, only values above 50% shown). Bar, 1 substitution per 100 nt positions.

CHu64-6-1^T (approximately 1,465 nt) was compared with the 16S rRNA gene sequences of the representative species within the genus *Flavihumibacter* and related genera. The novel strain showed the highest levels of 16S rRNA gene sequence similarity to *F. cheonanensis* KACC 17467^T, *F. petaseus* KACC 17385^T, *F. sediminis* KACC 18874^T, *F. solisilvae* KACC 17917^T, and *F. stibioxidans* KCTC 52205^T with similarities of 97.2%–98.1%. The levels of the 16S rRNA gene sequence similarity with the type strains of other members of the family *Chitinophagaceae* were less than 94%. However, a closely related species in this genus, *F. sediminis* sharing 98.3% pairwise similarity with *F. cheonanensis*, has been described recently (Lee and Cha, 2016). Strain CHu64-6-1^T also shared high similarities with other species than just members of *Flavihumibacter*, but it was evident from the topology of the phylogenetic tree (Fig. 1 and Supplementary data Fig. S1) that strain CHu64-6-1^T clustered clearly with the species of *Flavihumibacter*. To support this conclusion, the genomic delineation between the novel strain and the species of *Flavihumibacter* was supported by the DNA-DNA relatedness (the mean of triplicate experiments) data, for which strain CHu64-6-1^T shared DNA-DNA relatedness values of 12.4% with *F. cheonanensis* KACC 17467^T, 17.1% with *F. petaseus* KACC 17385^T, 33.2% with *F. sediminis* KACC 18874^T, 21.7% with *F. solisilvae* KACC 17917^T, and 15.1% with *F. stibioxidans* KCTC 52205^T. These values are well below the 70% cut-off point indicating the designation of the isolate as representative of a novel species (Wayne *et al.*, 1987).

Chemotaxonomic characteristics

The G + C content of the genomic DNA was determined to be 46.5 mol%. The major polar lipids were composed of phosphatidylethanolamine (PE), an unidentified phospholipid (PL1), and two unidentified aminolipids (AL1, AL2)

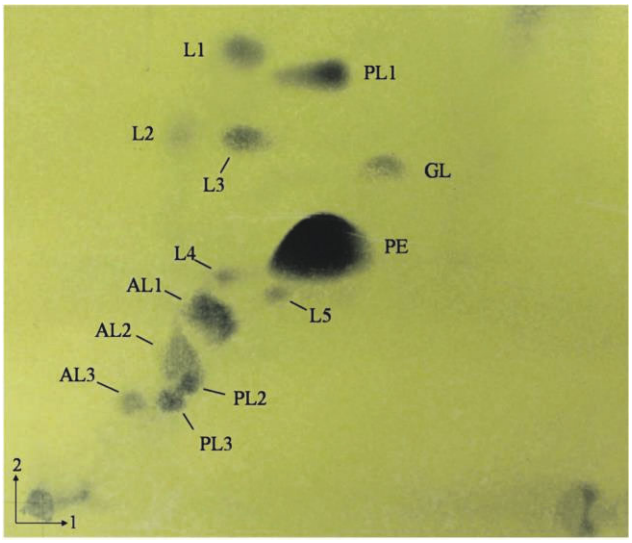

Fig. 2. Polar lipid profile for strain CHu64-6-1^T. AL, unidentified aminolipid; GL, unidentified glycolipid; L, unidentified lipids; PE, phosphatidylethanolamine; PL, unidentified phospholipid. 1, first dimension of TLC; 2, second dimension of TLC.

(Fig. 2). The major fatty acids were $C_{15:0}$ iso, $C_{17:0}$ iso 3-OH, $C_{15:1}$ iso G, and $C_{16:1}$ $\omega5c$. The major fatty acids in strain CHu64-6-1^T were consistent with the major fatty acid components in the species from the genus *Flavihumibacter* although there were differences in the proportions of some fatty acids (Table 2).

Based on the morphological, physiological (Table 1) and phylogenetic characteristics, our new isolate could be considered to be a member of the genus *Flavihumibacter*. Some physiological characteristics were found differentiating the novel strain from its closest formally described relatives. Especially, strain CHu64-6-1^T could be differentiated from all members in this genus by not assimilating *N*-acetyl-glucosamine, L-arabinose, maltose, D-melibiose, salicin, or D-sucrose. Therefore, we suggest that strain CHu64-6-1^T represents a novel species of the genus *Flavihumibacter*, for which the name *Flavihumibacter profundi* sp. nov. is proposed.

Description of *Flavihumibacter profundi* sp. nov.

Flavihumibacter profundi (pro.fun′di. L. gen. neut. n. *profundi* of a deep place).

Cells are Gram-stain-positive, non-motile, and rod-shaped. Colonies grown on R2A agar are yellow in color. Cells are oxidase-positive but catalase-negative. Growth occurs on R2A at temperatures from 15 to 37°C (optimum temperature 30°C), but not at 8 or 42°C. The pH range of growth is from pH 5.0 to 8.0 (optimum pH 7) but not at pH 4.0 and 9.0. Cells are positive for aesculin and gelatin hydrolysis, but negative for nitrate reduction, indole production, glucose acidification, arginine dihydrolase, or urease (API 20NE test strip). Cells assimilate succinic acid, but does not assimilate rest of carbon sources of test trips of API 20NE, API ID 32GN and Biolog GN2 microplates. Cells are positive for the following enzyme activities (API ZYM test strip): *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase. Cells arenegative for the following enzyme activities: α-chymotrypsin, lipase (C14), and β-glucuronidase. The cells are susceptible to amikacin (30 μg/ml), ampicillin (10 μg/ml), amoxycillin (10 μg/ml), cefadroxil (30 μg/ml), cefoperazone (75 μg/ml), chloramphenicol-C (30 μg/ml), ciprofloxacin (5 μg/ml), co-trimoxazole (25 μg/ml), erythromycin (15 μg/ml), gentamicin (10 μg/ml), nalidixic acid (10 μg/ml), netillin (30 μg/ml), nitrofurantoin (300 μg/ml), norfloxacin (10 μg/ml), penicillin (10 units), tobramycin (10 μg/ml), and vancomycin (30 μg/ml) but resistant to ceftazidime (30 μg/ml), ceftriaxone (30 μg/ml), and cloxacillin (1 μg/ml). The only respiratory quinone is MK-7. The major polar lipids are phosphatidylethanolamine, an unidentified phospholipid, and two unidentified aminolipids. The major fatty acids are $C_{15:0}$ iso, $C_{17:0}$ iso 3-OH, $C_{15:1}$ iso G, and $C_{16:1}$ $\omega5c$. The G + C content of the DNA is 46.5 mol%.

The type strain CHu64-6-1^T (= KCTC 62290^T = CCTCC AB 2018060^T) was isolated from a 67-cm-long sediment core taken from the Daechung Reservoir, Republic of Korea.

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