

Limibaculum halophilum gen. nov., sp. nov., a new member of the family *Rhodobacteraceae*

Yong Ho Shin,¹ Jong-Hwa Kim,¹ Ampaitip Suckhoom,² Duangporn Kantachote² and Wonyong Kim^{1,*}

Abstract

A Gram-stain-negative, cream-pigmented, aerobic, non-motile, non-spore-forming and short-rod-shaped bacterial strain, designated CAU 1123^T, was isolated from mud from reclaimed land. The strain's taxonomic position was investigated by using a polyphasic approach. Strain CAU 1123^T grew optimally at 37 °C and at pH 7.5 in the presence of 2 % (w/v) NaCl. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain CAU 1123^T formed a monophyletic lineage within the family *Rhodobacteraceae* with 93.8 % or lower sequence similarity to representatives of the genera *Rubrimonas*, *Oceanicella*, *Pleomorphobacterium*, *Rhodovulum* and *Albimonas*. The major fatty acids were C_{18:1} ω7c and 11-methyl C_{18:1} ω7c and the predominant respiratory quinone was Q-10. The polar lipids were phosphatidylethanolamine, phosphatidylglycerol, two unidentified phospholipids, one unidentified aminolipid and one unidentified lipid. The DNA G+C content was 71.1 mol%. Based on the data from phenotypic, chemotaxonomic and phylogenetic studies, it is proposed that strain CAU 1123^T represents a novel genus and novel species of the family *Rhodobacteraceae*, for which the name *Limibaculum halophilum* gen. nov., sp. nov. The type strain is CAU 1123^T (=KCTC 52187^T, =NBRC 112522^T).

The family *Rhodobacteraceae* was first established by Garrity *et al.* [1] as a member of the class *Alphaproteobacteria* (order *Rhodobacterales*, phylum *Proteobacter*). The names were validated in Validation List 107 [2]. The class *Alphaproteobacteria* contains the most abundant bacteria from marine environments and the family *Rhodobacteraceae* is also exclusively marine or hypersaline, with described isolates demonstrating salt tolerance [1, 3]. There are approximately 105 recognized genera in the family at the time of writing (www.bacterio.net/). Most members of the family originate from marine environments, such as seawater, sediments, marine snails, marine hot springs, marine phytoplankton and marine sponges. Only 14 genera, including *Actibacterium* [4], *Haslibacter* [5], *Huaishuia* [6], *Lentibacter* [7], *Oceanicella* [8], *Planktotalea* [9], *Poseidonocella* [10], *Defluviimonas* [11], *Epibacterium* [12], *Litorisediminicola* [13], *Pararhodobacter* [11], *Pelagimonas* [14], *Pleomorphobacterium* [15] and *Profundibacterium* [16], have been described in the last 5 years. In the course of screening the bacteria off the west coast of the Korean peninsula, strain CAU 1123^T was isolated from a mud sample from reclaimed land in Modo (37°32'12.28" N, 126°24'51.47" E), Republic of Korea. The aim of the study was to establish the taxonomic position of strain CAU 1123^T by using a polyphasic study that included the determination of phenotypic and

chemotaxonomic properties along with a detailed phylogenetic analysis based on 16S rRNA gene sequences.

Selective isolation of strain CAU 1123^T was performed according to Gordon and Mihm [17] by using the standard dilution plate technique. The appropriate dilutions were spread on marine agar 2216 (MA; Difco) plates and incubated under aerobic conditions at 30 °C for 10 days. A single colony of strain CAU 1123^T was purified by streaking several times until pure culture was formed on MA at 30 °C. The strain was preserved at –80 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (v/v) glycerol. Strain CAU 1123^T has been deposited in the Korean Collection for Type Cultures and the Biological Resource Centre, National Institute of Technology and Evaluation as KCTC 52187^T and NBRC 112522^T, respectively. The type strains of type species of the most closely related genera, *Rubrimonas cliftonensis* OCh317^T (=NBRC 100047^T), *Oceanicella actignis* PRQ-67^T (=DSM 22673^T), *Pleomorphobacterium xiame-nense* CLW^T (=DSM 24423^T), *Rhodovulum sulfidophilum* Hansen W4^T (=KCCM 41788^T) and *Albimonas donghaensis* DS^T (=KCTC 12586^T), were obtained from the National Institute of Technology and Evaluation, the Deutsche Sammlung von Mikroorganismen und Zellkulturen, the Korean Culture Centre of Microorganisms and the Korean

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Keywords: *Limibaculum halophilum*; *Rhodobacteraceae*; *Alphaproteobacteria*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1123^T is KX774334.

Three figures are available with the online Supplementary Material.

Collection for Type Cultures, and were used as reference strains for fatty acid and biochemical analyses

The 16S rRNA gene of strain CAU 1123^T was amplified using PCR with 27F and 1525R primers [18]. Multiple alignments with sequences of a broad selection of *Rhodobacteraceae* and calculation of sequence similarity levels were determined by using the CLUSTAL_X 2.1 program [19] and the EzTaxon-e server (<http://www.ezbiocloud.net>). Evolutionary distance matrices were created using the neighbour-joining method [20]. Phylogenetic trees were generated by using three different methods: the neighbour-joining [21], least-squares [22] and maximum-likelihood [23] algorithms in the PHYLIP package [24]. The bootstrap resampling values, based on 1000 replicates, were calculated using SEQBOOT and CONSENSE programs from the PHYLIP package [25]. The G+C content of the genomic DNA was determined as described by Tamaoka and Komagata [26] using reversed-phase high-performance liquid chromatography (HPLC). The G+C content was calculated by the ratio of deoxyguanosine to thymidine using standard DNAs according to the method of Mesbah *et al.* [27].

The nearly complete 16S rRNA gene sequence (1403 bp) was obtained and compared with reference sequences available in the GenBank database. Phylogenetic analysis based on the 16S rRNA gene showed that strain CAU 1123^T belonged to the family *Rhodobacteraceae* and formed a separate deep lineage with the recognized species of *Rubrimonas*, *Oceanicella*, *Pleomorphobacterium*, *Rhodovulum* and *Albimonas* (Fig. 1). The trees reconstructed by using the least-square, maximum-likelihood and maximum-parsimony algorithms presented a similar topology (data not shown). Strain CAU 1123^T showed very low levels of 16S rRNA gene sequence similarity to type strains of the most closely phylogenetically related species in the family *Rhodobacteraceae*: *R. cliftonensis* OCh317^T (similarity 93.8), *O. actignis* PRQ-67^T (similarity 93.0 %), *P. xiamenense* CLW^T (similarity 93.0 %), *Rhodovulum marinum* JA128^T (similarity 92.9 %) and *Albimonas pacifica* P-50-3^T (similarity 92.8 %). 16S rRNA gene sequence similarities to all other species of the family *Rhodobacteraceae* were below 92.8 %.

Strain CAU 1123^T and five reference strains, *R. cliftonensis* OCh317^T, *O. actignis* PRQ-67^T, *P. xiamenense* CLW^T, *R. sulfidophilum* Hansen W4^T, and *A. donghaensis* DS^T were

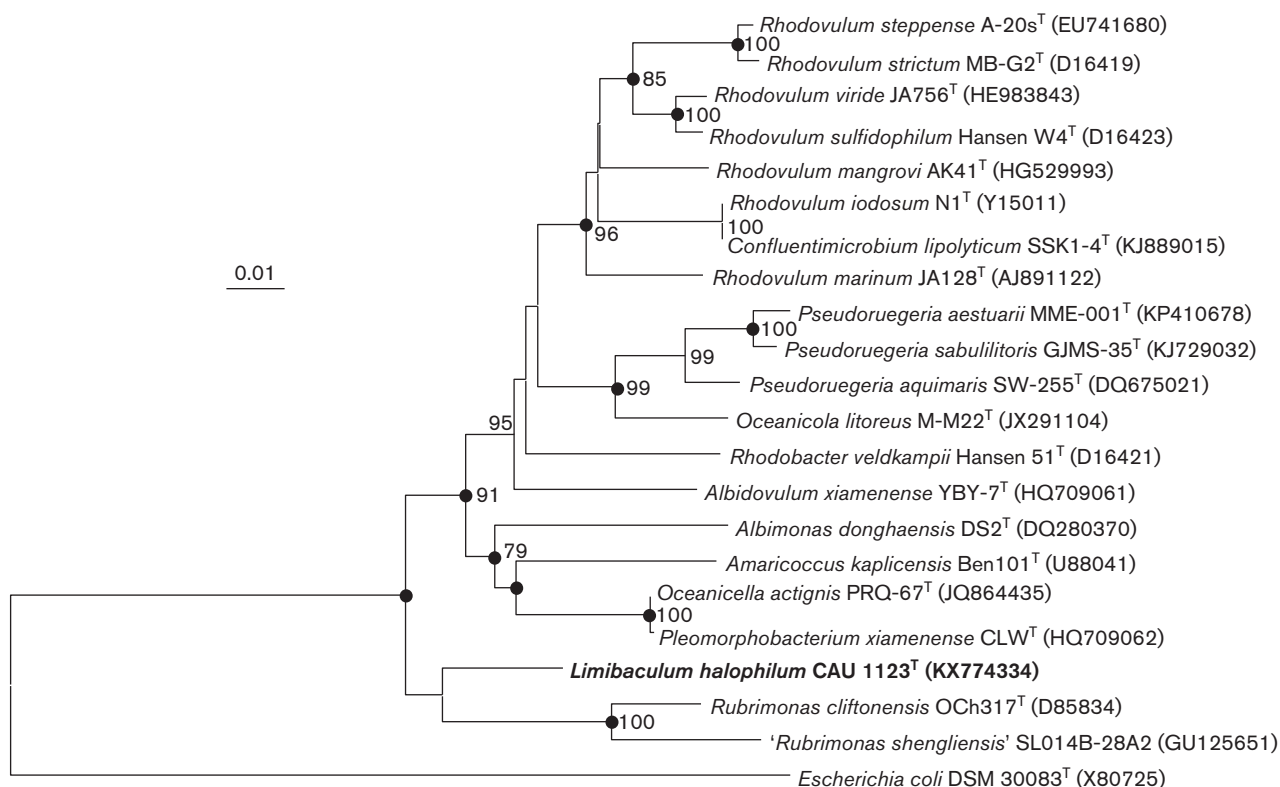


Fig. 1. Phylogenetic tree derived from nearly complete 16S rRNA gene sequences showing the position of strain CAU 1123^T among members of the family *Rhodobacteraceae*. The tree was reconstructed by using the neighbour-joining method and rooted *Escherichia coli* DSM 30083^T (X80725) as the outgroup. Dots indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and least-squares algorithms. Values of bootstrap (1000 resamplings) are indicated at the nodes and only values >70 % are shown. Bar, 0.01 substitutions per nucleotide position.

cultivated on MA at their optimal growth temperatures to determine their morphological, physiological and biochemical properties. Cell morphology was observed by light (DM 1000; Leica) and transmission electron (TEM; JEM 1010, JEOL) microscopy using cells from the exponential growth stage in MA at 37 °C. For TEM purpose, a small drop of bacterial suspension was put on a grid and stained with 1 % (w/v) phosphotungstic acid. Motility was examined by using MA soft agar (0.3 % agar) and gliding motility was evaluated with cells on microscopic slides coated with MA (0.7 % agar) using the hanging drop method [28]. The Gram reaction was tested using a Gram staining kit (bioMérieux). Oxidase activity was examined by 1 % (w/v) tetramethyl-*p*-phenylenediamine and catalase activity was determined by bubble production in 3 % hydrogen peroxide [29]. Growth at various temperatures (4–45 °C) was assessed using an aerobic incubator (MIR-253, Sanyo) and an anaerobic chamber (Bactron, Sheldon) using a streaked MA plate after 5 days

incubation. The pH value for growth was examined at 37 °C in MB adjusted within a pH range of 4.5–11.5 (at intervals of 0.5 pH unit) and values were confirmed again and adjusted using sodium acetate/acetic acid, Tris/HCl and Na₂CO₃ buffers after autoclaving. Growth with NaCl was investigated at 37 °C at a range of 0–15 % (w/v) by supplementing MB with an appropriate concentration of NaCl, which was prepared according to the formula of the BD medium except that NaCl was excluded. Hydrolysis of casein, starch and urea were tested as described by Lányi [30] and Smibert and Krieg [31]. Acid production from carbohydrates and other biochemical characteristics were examined using API 20E and API 20NE (after 24 h), and API 50CH (after 48 h; bioMérieux). Enzyme activities were investigated by using API ZYM (bioMérieux) after 24 h.

The detailed morphological, cultural, physiological and biochemical characteristics of strain CAU 1123^T are presented in Table 1 and in the species description. Strain

Table 1. Phenotypic characteristics of strain CAU 1123^T and the closest-related members of the family *Rhodobacteraceae*

Strains: 1, CAU 1123^T; 2, *Rubrimonas cliftonensis* OCh317^T; 3, *Oceanicella actignis* PRQ-67^T; 4, *Pleomorphobacterium xiamenense* CLW^T; 5, *Rhodovulum sulfidophilum* Hansen W4^T; 6, *Albimonas donghaensis* DS^T. Data were obtained in this study unless indicated. All taxa were positive for activity of alkaline phosphate, catalase and oxidase. All taxa were negative for activity of α -chymotrypsin and α -mannosidase. +, Positive; –, negative; w, weakly positive; ND, no data available. AL, unknown aminolipid; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

Characteristic	1	2	3	4	5	6
Habitat	Reclaimed land mud	Saline lake ^a	Shallow marine hot spring ^c	Terrestrial hot spring ^d	Marine ^e	Seawater ^g
Cell shape	Short rod	Short rod	Pleomorphic	Short rod	Ovoid or rod	Rod
Flagella	–	+	–	+	+	–
Temperature (°C)						
Range	20–40	27–30	25–57.5	28–65	25–35	10–38
Optimum	37	28	50	52	28	30
pH range						
Range	6.5–9.0	7.5–8.0 ^a	6.0–9.0 ^c	5.5–9.5 ^d	5.0–9.0 ^e	6.0–9.5 ^g
Optimum	7.5	7.5	7.5	7.0	7.0	7.5
NaCl tolerance (% w/v)						
Range	0–7	0.5–7.5	1–9	0.3–6	0–10	0–14
Optimum	2	2	3	3	2	2
Nitrate reduction	+	+	+	–	–	–
Hydrolysis of						
Gelatin	+	–	+	+ ^d	–	+
Citrate	+	+	–	–	–	+
Sodium pyruvate	–	–	+	–	+	+
Utilization of carbon sources						
L-Arabinose	–	+	+	w	w	+
D-Ribose	–	+	+	+	w	–
D-Xylose	–	+	+	w	w	w
D-galactose	–	+	–	–	+	–
D-Dlucose	–	+	–	–	+	–
D-Fructose	–	+	+	–	–	–
Major polar lipids	PG, PE	PG, PC ^b	PC, PG, 1AL ^c	PG, PE, PC ^d	PG, PE ^f	PG, DPG, PE, PC ^g
DNA G+C content (mol%)	71.1	74.0–74.8 ^a	71.2 ^c	72.2 ^d	66.3–66.6 ^e	72.0 ^g

*Data taken from: a, Suzuki et al. [38]; b, Cai et al. [46]; c, Albuquerque et al. [8]; d, Yin et al. [15]; e, Srinivas et al. [47]; f, Ramaprasad et al. [44]; g, Li et al. [48].

CAU 1123^T was Gram-stain-negative, aerobic, non-spore forming and non-motile. Cells were rod-shaped and approximately 0.6–1.3 µm in diameter and 1.2–1.9 µm in length. Colonies were cream coloured, circular and slightly convex with diameters of 0.2–0.3 mm after 3 days of cultivation on MA at 30 °C. Flagella were not observed (Fig. S1, available with the online Supplementary Material). This characteristic is different from its relative genera, *Rubrimonas* and *Pleomorphobacterium*. Growth of strain CAU 1123^T was observed at temperatures between 20 and 40 °C (optimum, 37 °C) and at pH 6.5–9.0 (optimum, pH 7.5) in the presence of 0–7 % (w/v) NaCl (optimum 2 %). The growth temperature range distinguished strain CAU 1123^T (20–40 °C) from the genera *Pelagibius* (15–33 °C), *Fodinicurvata* (15–42 °C), *Rhodovibrio* (25–47 °C), *Nisaea* (15–44 °C), *Thalassobaculum* (10–35 °C) and *Azospirillum* (4–41 °C). In addition, the salt tolerance range distinguished strain CAU 1123^T (0–7 %) from the genera *Pelagibius* (2–6 %), *Fodinicurvata* (1.5–20), *Rhodovibrio*

(3–24 %) and *Thalassobaculum* (1–10 %). Strain CAU 1123^T hydrolysed gelatin and had enzyme activities for alkaline phosphate, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, Naphtol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. Citrate was utilized. Xylitol, D-tagatose, L-fructose, D-arabitol and potassium 5-keto-cluconate were utilized as sole carbon and energy sources, but L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, lactose, D-ribose and D-mannitol were not utilized. Compared with its closest relatives, *R. cliftonensis* OCh317^T, *O. actignis* PRQ-67^T, *P. xiamenense* CLW^T, *R. sulfidophilum* Hansen W4^T and *A. donghaensis* DS^T, strain CAU 1123^T differed by having a positive reaction in the test for *N*-acetyl-β-glucosaminidase.

Chemotaxonomic properties of CAU 1123^T and five reference strains were cultured and compared under the same conditions. For fatty acid analysis, all strains were harvested

Table 2. Cellular fatty acid content (%) of strain CAU 1123^T and the most closely related genera of the family *Rhodobacteraceae*

Strains: 1, CAU 1123^T; 2, *Rubrimonas cliftonensis* OCh317^T; 3, *Oceanicella actignis* PRQ-67^T; 4, *Pleomorphobacterium xiamenense* CLW^T; 5, *Rhodovulum sulfidophilum* Hansen W4^T; 6, *Albimonas donghaensis* DS^T. Data were obtained in this study. +, Positive; TR, trace (<0.5 %); –, not detected.

Fatty acids	1	2	3	4	5	6
Saturated						
C _{14:0}	–	0.7	–	–	–	2.4
C _{16:0}	–	16.2	2.1	1.0	13.3	6.0
C _{17:0}	1.8	TR	1.7	0.5	TR	TR
C _{18:0}	0.9	2.8	8.1	3.4	4.2	7.2
C _{18:1}	–	58.9	–	–	73.0	–
Unsaturated						
C _{18:1} ω7c	69.5	TR	56.4	87.2	2.3	63.2
C _{20:1} ω7c	–	–	0.7	–	–	–
C _{20:1} ω9c	–	–	TR	–	–	–
11-methyl C _{18:1} ω7c	25.5	10.3	23.0	TR	TR	12.8
10-methyl C _{19:0}	–	–	–	0.9	–	–
Branched chain						
Anteiso-C _{17:1} ω9c	–	–	TR	–	–	–
Iso-C _{15:0}	–	0.9	–	–	–	–
Iso-C _{13:0} 3-OH	–	–	0.7	TR	–	–
Iso-C _{16:0} 3-OH	–	TR	–	–	–	–
Iso-C _{15:1} F	–	–	0.6	TR	–	–
Hydroxy						
C _{10:0} 3-OH	–	–	–	2.4	2.6	–
C _{14:0} 3-OH	–	–	–	–	1.5	–
C _{18:0} 3-OH	1.5	4.3	2.0	3.6	–	4.2
*Summed feature						
2	–	3.0	3.6	–	–	4.0
3	0.6	2.5	–	TR	2.9	–
4	–	–	TR	–	–	–
7	TR	–	–	–	–	–

*Summed features represent groups of two or three fatty acids that could not separate by gas–liquid chromatography with the MIDI system. Summed feature 2 contained unknown 10.928, summed feature 3 contained C_{16:1} ω7c and/or C_{16:1} ω6c, summed feature 4 contained anteiso-C_{17:1} B and/or iso I, and summed feature 7 contained C_{19:1} ω6c and/or unknown 18.846.

from MA after cultivation at 37 °C for 3 days. The physiological age of the bacterial cell was normalized by observing growth development during incubation of the cultures and choosing the moment of harvesting according to the standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Fatty acids were extracted using the method of Minnikin *et al.* [32] and separated by using a 6890 N automated gas chromatography system (Agilent Technologies) and identified by using the Microbial Identification software package (MOORE library version 5.0; MIDI database TSA6). The polar lipids of strain CAU 1123^T were analysed by two-dimensional thin-layer chromatography (TLC) according to the method of Minnikin *et al.* [33]. The plate was sprayed with various staining reagents: 10 % ethanolic molybdato-phosphoric acid (for the total lipids); molybdenum blue (for phospholipids); ninhydrin (for aminolipids) and α -naphthol/sulphuric acid reagent (for glycolipids; Sigma-Aldrich). Isoprenoid quinone was extracted according to Komagata and Suzuki [34] and separated by HPLC using an isocratic solvent system [methanol/isopropyl ether (3:1, v/v)] and a flow rate of (1 ml min⁻¹).

The fatty acid profile of strain CAU 1123^T is shown Table 2. The major fatty acids were C_{18:1}ω7c and 11-methyl C_{18:1}ω7c. C_{18:1}ω7c is characteristic fatty acid of the type strains of *P. xiamenense* CLW^T and *A. donghaensis* DS^T. While, 11-methyl C_{18:1}ω7c is most abundant in *O. actignis* PRQ-67^T. The small amount of fatty acid also contained iso-C_{14:0}, C_{14:0}, iso-C_{13:0} 3-OH, iso-C_{15:1} F, anteiso-C_{15:1} A, iso-C_{15:0}, C_{16:0}N alcohol, iso-C_{16:0}, C_{16:0}, anteiso-C_{17:1}ω9c, iso-C_{17:0} and anteiso-C_{17:0}, C_{17:0}, C_{18:0}. The polar lipid profile of strain CAU 1123^T included phosphatidylethanolamine and phosphatidylglycerol as major polar lipids and two unidentified phospholipids, one unidentified aminolipid and one unidentified lipid (Figs S2 and S3). This polar lipid pattern was different the most closely related members of the family *Rhodobacteraceae*, *R. cliftonensis* OCh317^T, *O. actignis* PRQ-67^T, *P. xiamenense* CLW^T, *R. sulfidophilum* Hansen W4^T and *A. donghaensis* DS^T by the absence of phosphatidylcholine. Strain CAU 1123^T contained ubiquinone 10 (Q-10), as has been found in other closely related species of the family *Rhodobacteraceae* [35–45].

DESCRIPTION OF LIMIBACULUM GEN. NOV.

Limibaculum (Li.mi.ba'cu.lum. L. n. *limus* mud; L. neut. n. *baculum* stick; N.L. neut. n. *Limibaculum*, a rod-shaped bacterium from a mud).

Cells are aerobic, short-rods which do not form spores. Catalase- and oxidase-positive. The Gram reaction is negative. Q-10 is the predominant ubiquinone and C_{18:1}ω7c and 11-methyl C_{18:1} are the main fatty acids. Phylogenetically, 16S rRNA gene sequence analysis classifies the genus *Limibaculum* in the family *Rhodobacteraceae*. The type species is *Limibaculum halophilum*.

DESCRIPTION OF LIMIBACULUM HALOPHILUM SP. NOV.

Limibaculum halophilum (ha.lo'phi.lum. Gr. n. *hals*, *halos* salt; Gr. adj. *philos* loving; N.L. neut. adj. *halophilum* salt loving referring to the requirement for salt).

Cells are Gram-stain-negative, non-motile and short rods approximately 0.6–1.3 μm in diameter and 1.2–1.9 μm in length. Optimum growth occurs at 37 °C and at pH 7.5 with 2 % (w/v) NaCl. Gelatin and aesculin are hydrolysed, but casein, starch and urea are not. Xylitol, D-tagatose, L-fucose, D-arabitol and potassium 5-ketocluconate are utilized as a sole carbon and energy sources, but L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, lactose, D-ribose and D-mannitol are not. According to API ZYM tests, it is positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase. In API 20NE tests, it is positive for nitrate reduction and trisodium citrate, but negative for L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid and phenylacetic acid. The DNA G+C content is 71.1 mol%. The type strain is CAU 1123^T (=KCTC 52187^T, =NBRC 112522^T), isolated from mud from reclaimed land in Mado in South Korea.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The authors have declared that no ethical issues exist.

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