

Nonlabens halophilus sp. nov., isolated from reclaimed land

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Abstract

A Gram-stain-negative, orange-pigmented, non-spore-forming, non-motile, aerobic, rod-shaped bacterial strain, designated CAU 1131^T, was isolated from reclaimed land. Strain CAU 1131^T grew optimally at 30 °C and at pH 6.5 in the presence of 4 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain CAU 1131^T was grouped into the genus *Nonlabens*, and was most closely related to *Nonlabens. marinus* S1-08^T (95.9 % 16S rRNA gene sequence similarity). The strain possessed MK-6 as the predominant menaquinone and iso-C_{15:0}, iso-C_{15:0} 3-OH, iso-C_{17:0} 3-OH and summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c) as the major cellular fatty acids. The polar lipid profile was determined to comprise phosphatidylethanolamine, phosphatidylcholine, an unidentified aminolipid, an unidentified glycolipid and two unidentified lipids. The DNA G+C content was 38.7 mol%. On the basis of data from phenotypic, chemotaxonomic and phylogenetic inference, strain CAU 1131^T represents a novel species of the genus *Nonlabens*, for which the name *Nonlabens halophilus* sp. nov. is proposed. The type strain is CAU 1131^T (=KCTC 52177^T=NBRC 111996^T).

The genus *Nonlabens*, a member of the family *Flavobacteriaceae* of the phylum *Bacteroidetes* [1], was proposed by Lau et al. [2] with the description of a single novel species, *Nonlabens tegetincola*, as the type species of the genus. The genus *Nonlabens* consists of Gram-stain-negative, aerobic, non-motile, non-spore-forming, short rod-shaped bacteria that are characterized by the presence of menaquinone-6 (MK-6) as the predominant respiratory quinone [3]. At the time of writing, this genus comprises 10 species with validly published names [4]: *Nonlabens tegetincola* [3], *Nonlabens dokdonensis* [5, 6], *Nonlabens xylanidelens* [7], *Nonlabens sediminis* [8], *Nonlabens spongiae* [9], *Nonlabens ulvanivorans* [10], *Nonlabens marinus* [11], *Nonlabens agnitus* [2], *Nonlabens arenilitoris* [12] and *Nonlabens antarcticus* [13]. Members of the genus *Nonlabens* have been isolated from marine environments such as tissue of marine sponge [9], seawater [2, 11], glacier core [13] and marine sand [12]. The novel bacterial strain, designated CAU 1131^T, was isolated from a reclaimed land soil sample collected in Modo (37° 32' 12.28" N 126° 24' 51.47" E) in the Republic of Korea. In this study, we performed a polyphasic characterization of strain CAU 1131^T to determine the taxonomic position of this bacterial strain by using phenotypic and chemotaxonomic properties, and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Isolation was performed according to Gordon and Mihm [14] using marine agar 2216 (MA; Difco). The crushed soil sample was serially diluted with sterilized 0.9 % sodium chloride

solution and plated on MA. The agar plates were incubated under aerobic conditions at 30 °C for 7 days. Strain CAU 1131^T was purified by subculture plating on MA. The strain was well-maintained at –70 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (v/v) glycerol. The type strains of the most closely related species, *N. marinus* S1-08^T (=KCTC 23432^T), *N. antarcticus* AKS622^T (=JCM 14068^T), *N. agnitus* JC2678^T (=KACC 14155^T) and *N. spongiae* UST030701-156^T (=JCM 13191^T) were obtained from the Korean Agricultural Culture Collection (KACC; Suwon, Korea), the Korean Collection for Type Cultures (KCTC; Jeonjeup, Korea), and the Japan Collection of Microorganisms (JCM; Tsukuba, Japan), and were used as reference strains.

Genomic DNA of strain CAU 1131^T was extracted and purified using a genomic DNA extraction kit (Intron). Amplification of the 16S rRNA gene was performed by PCR with the universal primers 8F/1525R following established procedures [15]. The 16S rRNA gene amplicon was sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an automatic DNA sequencer (model 3730; Applied Biosystems). Multiple alignments and calculation of 16S rRNA gene sequence similarity between strain CAU 1131^T and other closely related strains were conducted by using EzTaxon (www.ezbiocloud.net) and CLUSTAL X 2.1 software [16]. Phylogenetic trees were reconstructed by using three algorithms: distance analyses with maximum-likelihood ([17]; DNAML program from the PHYLIP 3.66 package),

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neighbour-joining [18] and Fitch–Margoliash ([19]; also from PHYLIP). The distance matrix was produced on the basis of the Jukes–Cantor model [20]. The tree topology was evaluated by the bootstrap resampling method [21] with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package. The mol% G+C content of the genomic DNA was determined using HPLC by the method of Tamaoka and Komagata [22].

The almost-complete 16S rRNA gene sequence of strain CAU 1131^T (1504 bp) was determined and compared with the available reference sequences in the GenBank database (accessed July 2016). Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain was affiliated to the genus *Nonlabens*. The neighbour-joining tree is shown in Fig. 1. The trees obtained with the two other treeing methods used showed essentially the same topology (data not shown). In the neighbour-joining phylogenetic tree, strain CAU 1131^T formed cluster with *N. spongiae* UST030701-156^T. Strain CAU 1131^T showed the highest 16S rRNA gene sequence similarity to *N. marinus* S1-08^T (similarity, 95.9%) followed by type strains of nine recognized species of the genus *Nonlabens* (94.9–94.0%). The G+C content of the DNA of strain CAU 1131^T was 38.7 mol%.

Strain CAU 1131^T was cultivated on MA at 30 °C to investigate all morphological, biochemical and physiological characteristics [23], except for spore formation that was assessed on nutrient sporulation medium [24]. Cell morphology was observed by light microscopy (DM 1000; Leica). Transmission electron microscopy (JEM 1010; JEOL) was used to examine the presence of flagella on cells from an exponentially growing culture. Gram staining was performed using the bioMérieux Gram staining kit. Gliding motility was evaluated using the hanging-drop method as described by Bowman [25]. The temperature range for growth of strain CAU 1131^T in MA was determined by measuring the turbidity of the broth after 72 h at 4, 10, 20, 30, 37, 45 and 55 °C in an aerobic incubator (MIR-253; Sanyo) and in an anaerobic chamber (Bactron; Sheldon). Growth was tested at 30 in MB adjusted to pH 4.5–11.0 at 0.5 pH unit intervals. The pH values of <6, 6–9 and >9 were obtained by using sodium acetate/acetic acid, Tris/HCl and sodium carbonate buffers, respectively. Growth in the absence of NaCl and in the presence of 1–15.0% (w/v) NaCl (at increments of 1% intervals) was examined at 30 °C in MB prepared according to the formula of the Difco medium, except that NaCl was excluded and that 0.45% (w/v) MgCl₂ · 6H₂O and 0.06% (w/v) KCl were added. Catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined by the oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine [26]. Hydrolysis of casein, aesculin and gelatin, and nitrate reduction were determined according to Smibert and Krieg [27]. Acid production from carbohydrates and other biochemical and physiological features were investigated using the API 20NE, 20E and 50CH systems (bioMérieux). Enzyme activities were examined using the API ZYM system (bioMérieux) according to the manufacturer's instructions. API 20NE and

50CH strips were inoculated with API AUX and CHE/B medium supplemented to 2% NaCl. API 20E and API ZYM strips were used with bacterial suspensions in 2% NaCl solution. API 20E, API 20NE and API 50CH test strips were read after incubation for 48 h at 30 °C and the API ZYM test strip was read after incubation for 24 h at 37 °C. Flexirubin-type pigments were examined by using the bathochromatic shift test with 20% (w/v) KOH solution [23]. The gene sequence of proteorhodopsin was confirmed using one primer set (PR-Flavo-F and PR-Flavo-R) reported by Yoshizawa *et al.* [28]. Susceptibility to antibiotics was tested on MA plates using Sensi-Disc susceptibility test discs (BBL) containing the following antibiotics (µg per disc unless otherwise stated): amoxicillin (20), ampicillin (10), carbenicillin (100), cefoxitin (30), cephalothin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), penicillin (10 U), nalidixic acid (30), polymyxin B (300 U), rifampin (5), streptomycin (10), tetracycline (30), tobramycin (10) and trimethoprim/sulfamethoxazole (1.25/23.75).

Detailed phenotypic characteristics of strain CAU 1131^T are given in the species description and in Table 1. Strain CAU 1131^T was Gram-stain-negative, aerobic, non-motile and non-spore-forming. Cells were rod-shaped approximately 0.1–0.4 µm in diameter and 1.0–3.0 µm in length. Colonies were orange, circular, and convex with entire margins after 3 days on MA at 30 °C. Flagella were not observed (Fig. S1, available in the online Supplementary Material). Growth of strain CAU 1131^T was observed at 10–37 °C (optimum 30 °C) and pH 5.5–10.5 (optimum pH 6.5) in the presence of 0–5% (w/v) NaCl (optimum 4%). Strain CAU 1131^T hydrolysed gelatin and aesculin, but not casein and starch. Strain CAU 1131^T was susceptible to all antibiotics that were tested. Flexirubin-type pigments were not produced. The isolate did not contain proteorhodopsin. However, some phenotypic characteristics of strain CAU 1131^T were distinct among the species of the genus *Nonlabens* including positive reactions for acid production from D-lyxose and D-tagatose, and for utilization of D-lyxose, D-tagatose and D-ribose. In addition, the negative reactions for utilization of D-glucose and enzyme activities of leucine arylamidase, cystine arylamidase and trypsin showed distinction between strain CAU 1131^T and the species of the genus *Nonlabens*. These differences were adequate to suggest that strain CAU 1131^T was different from other species of the genus *Nonlabens*. Strain CAU 1131^T had representative characteristics of the genus *Nonlabens* including the presence of alkaline phosphatase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities in the API ZYM strip, and positive results for inositol, D-sorbitol and L-rhamnose in the API 20E strip. Strain CAU 1131^T differed from closely related species, namely *N. marinus* KCTC 23432^T by its negative reaction for D-mannitol, utilization of D-glucose, and enzyme activities of leucine arylamidase and trypsin, and KCTC 23432^T by its positive reaction for esterase (C4) and lipase (C14) activities. Based on the comparison of phenotypic data obtained in this study (Table 1), strain CAU 1131^T could be distinguished from the recognized species of the genus *Nonlabens*.

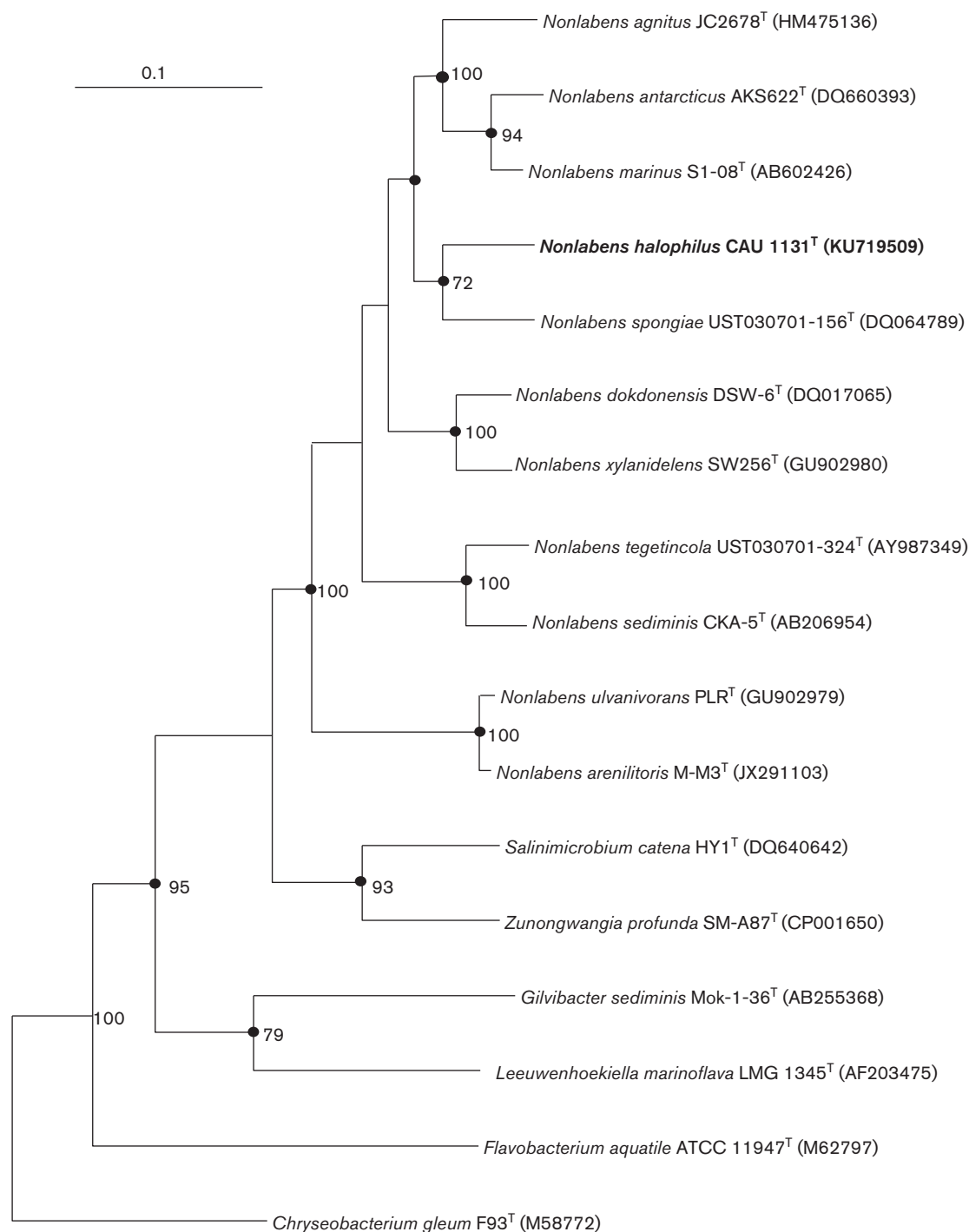


Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences, showing the relationships between strain CAU 1131^T and the type strains of reference species of the genus *Nonlabens*. Filled circles indicate that the corresponding nodes were also recovered in the trees created with the least-squares and maximum-likelihood algorithms. Bootstrap values are indicated as percentages of 1000 resampled datasets, when >70 %. *Chryseobacterium gleum* F93^T (GenBank accession number M58772) is used as an outgroup organism. Bar, 0.1 substitutions per nucleotide position.

For cellular fatty acid analysis, the cell mass of strain CAU 1131^T and the type strains of the most closely related species of the genus *Nonlabens*, *N. marinus* KCTC 23432^T, *N.*

antarcticus JCM 14068^T, *N. agnitus* KACC 14155^T and *N. spongiae* JCM 13191^T, were harvested from MA in late-exponential growth phase after cultivation for 3 days at

37 °C according to a standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Fatty acid methyl esters were obtained as described by Minnikin *et al.* (29), and separated by an automated gas chromatography system (model 6890N and 7683 autosampler; Agilent). The peaks were detected by the Microbial Identification software package (MOORE library version 5.0; MIDI database TSBA6). Respiratory quinones were extracted and analysed as described previously [30] using HPLC. For analysis of polar lipids, cells of strain CAU 1131^T were harvested from MA after cultivation for 3 days at 30 °C. The polar lipids of strain CAU 1131^T were identified using two-dimensional thin-layer chromatography (silica gel 60 F254, 20×20 cm plates; Merck) by the method of Minnikin *et al.* [31]. The plates were sprayed with 10 % ethanolic molybdotophosphoric acid (for detection of total lipids), molybdenum blue (for phospholipids), α -naphthol/sulphuric acid reagent (for glycolipids), ninhydrin (for aminolipids), and Dragendorff's reagent (for phosphatidylcholine) (Sigma-Aldrich).

MK-6 was detected as the only respiratory quinone in strain CAU 1131^T. This feature is compatible with the genus *Nonlabens*, including the type strain of the type species of the genus, *N. tegetincola* UST030701-324^T. The cellular fatty acid profiles of strain CAU 1131^T, *N. marinus* KCTC 23432^T, *N. antarcticus* JCM 14068^T, *N. agnitus* KACC 14155^T and *N. spongiae* JCM 13191^T are shown in Table 2. The major cellular fatty acids of strain CAU 1131^T were found to be iso-C_{15:0} (14.12 %), iso-C_{15:0} 3-OH (7.30 %), iso-C_{17:0} 3-OH (17.84 %) and summed feature 3 (comprising C_{16:1} ω 7c and/or C_{16:1} ω 6c, 17.71 %) which are characteristic elements of the fatty acid profiles of the type strains of species of the genus *Nonlabens* (Table 2). Despite the similarity in the fatty acid composition of strain CAU 1131^T and the reference strains, there were differences in the amounts of some fatty acids. Furthermore, strain CAU 1131^T could be distinguished from closely related species by higher percentages of iso-C_{17:1} ω 9c and summed feature 3 (comparing C_{16:1} ω 7c and/or C_{16:1} ω 6c). The major polar lipids of strain CAU 1131^T comprised phosphatidylethanolamine and phosphatidylcholine, and one aminolipid, one glycolipid and two lipids as unidentified polar lipids (Fig. S2). The polar lipids profile of strain CAU 1131^T was similar to those of the type strains of species of the genus *Nonlabens* in that phosphatidylethanolamine was a major polar lipid.

Therefore, these data from the phenotypic, chemotaxonomic and phylogenetic study provide sufficient evidence to recognize strain CAU 1131^T as a novel species of the genus *Nonlabens*, for which the name *Nonlabens halophilus* sp. nov. is proposed.

DESCRIPTION OF *NONLABENS HALOPHILUS* SP. NOV.

Nonlabens halophilus (ha.lo'phi.us. Gr. n. *hals*, *halos* salt; Gr. adj. *philos* friendly, loving N.L. masc. adj. *halophilus* salt-loving referring to the requirement for salt).

Table 1. Differential properties of strain CAU 1131^T and the type strains of the most closely related species of the genus *Nonlabens*

Strains: 1, CAU 1131^T; 2, *N. marinus* KCTC 23432^T; 3, *N. antarcticus* JCM 14068^T; 4, *N. agnitus* KACC 14155^T; 5, *N. spongiae* JCM 13191^T. Data were taken from this study unless otherwise indicated. All strains were positive for oxidase, hydrolysis of gelatin, and alkaline phosphatase, esterase lipase (C8), valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities. All strains were negative for assimilation of D-glucose, L-arabinose, D-mannose, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, malic acid, trisodium citrate, phenylacetic acid, erythritol, L-xylose, D-adonitol, L-sorbose, dulcitol, methyl β -D-xylopyranoside, D-melezitose, xylitol, D-fucose, L-fucose, D-arabitol, L-arabitol and potassium 2-ketogluconate. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Gliding motility	–	–	+	+	+
Temperature for growth (°C)					
Range	10–37	10–30 ^a	8–20 ^b	4–35 ^c	20–36 ^d
Optimum	30	25 ^a	15 ^b	30 ^c	28–30 ^d
pH for growth					
Range	6.5–10	6–9 ^a	6.5–8.5 ^b	5.5–9 ^c	6–10 ^d
Optimum	6.5	7–8 ^a	7 ^b	7 ^c	7
NaCl (% w/v)	4	2–3 ^a	2 ^b	2–3 ^c	2–6 ^d
Catalase	+	+	+	+	+
API 20NE tests					
D-Mannitol	–	+	–	–	+
Adipic acid	–	–	+	–	–
API 20E tests					
Sucrose	–	–	+	+	–
API ZYM tests					
Esterase (C4)	+	–	–	–	+
Lipase (C14)	+	–	–	–	+
Leucine arylamidase	–	+	+	+	+
Cystine arylamidase	–	+	+	+	+
Trypsin	–	+	+	+	+
Acid production from:					
D-Lyxose	+	–	–	–	+
D-Tagatose	+	–	–	–	+
Sucrose	–	+	–	+	–
Utilization of carbon sources					
Sucrose	–	+	–	+	–
D-Glucose	–	+	+	+	+
D-Mannitol	–	+	–	–	+
D-Lyxose	+	–	–	–	+
D-Tagatose	+	–	–	+	+
D-Ribose	+	–	–	+	–
Hydrolysis of aesculin	+	+	–	–	–
DNA G+C content (mol %)	38.7	41.7 ^a	37.9 ^b	37 ^c	41.0 ^d

*Data from: a, Park *et al.* [11]; b, Kwon *et al.* [13]; c, Yi and Chun [2]; d, Lau *et al.* [9].

Table 2. Cellular fatty acid contents (%) of strain CAU 1131^T and the type strains of the most closely related species of the genus *Nonlabens*

Strains: 1, CAU 1131^T; 2, *N. marinus* KCTC 23432^T; 3, *N. antarcticus* JCM 14068^T; 4, *N. agnitus* KACC 14155^T; 5, *N. spongiae* JCM 13191^T. All data are from this study. —, Not detected; TR, trace (<1 %).

Fatty acid	1	2	3	4	5
Saturated					
C _{16:0}	1.05	TR	TR	1.59	—
Unsaturated					
C _{15:1} ω6c	1.31	TR	1.22	—	1.28
C _{17:1} ω6c	2.70	4.86	3.72	1.03	1.11
Branched					
anteiso-C _{15:0}	5.67	10.03	24.80	6.10	4.87
anteiso-C _{17:1} ω9c	3.84	2.54	4.74	—	—
iso-C _{15:0}	14.12	12.49	3.66	27.12	35.88
iso-C _{16:0}	1.55	13.20	9.14	10.77	1.13
iso-C _{16:1} H	1.38	2.74	3.64	—	—
iso-C _{15:0} 3-OH	7.30	2.30	TR	4.36	9.84
iso-C _{16:0} 3-OH	4.56	12.55	10.46	5.29	2.65
iso-C _{17:0} 3-OH	17.84	10.77	3.13	20.78	17.30
iso-C _{17:1} ω9c	4.55	3.27	1.71	3.06	2.82
Hydroxyl					
C _{15:0} 2-OH	2.99	1.93	3.45	1.24	1.43
C _{16:0} 3-OH	2.63	1.49	1.95	1.15	TR
C _{17:0} 2-OH	5.27	6.02	8.02	4.24	2.21
Summed feature 3*	17.71	8.13	11.65	4.65	9.88

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or C_{16:1}ω6c.

Cells are Gram-stain-negative, non-motile, anaerobic, non-spore-forming and rod-shaped, approximately 0.1–0.4 μm in diameter and 1.0–3.0 μm in length. Colonies grown on MA after incubation for 3 days at 30 °C are orange, circular and convex with entire margins. Growth occurs at 10–37 °C (optimum 30 °C), at pH 5.5–10.5 (optimum pH 6.5) and with 0–5 % (w/v) NaCl (optimum 2 %). Catalase and oxidase are present. Gelatin is liquefied. Utilizes D-mannitol in the API 20E system. In addition, inositol, D-sorbitol and L-rhamnose are assimilated in the API 20NE system. Utilizes D-ribose, D-xylose, D-lyxose, D-tagatose and potassium 5-keto-gluconate. In the API ZYM strip, alkaline phosphatase, esterase (C4), lipase (C14), esterase lipase (C8), valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphatase activities are present, but leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The major polar lipids are phosphatidylethanolamine, phosphatidylcholine, an unidentified aminolipid, an unidentified glycolipid and two unidentified lipids. The major isoprenoid quinone is MK-6. The main fatty acids detected are iso-C_{15:0}, iso-C_{15:0} 3-OH,

iso-C_{17:0} 3-OH and summed feature 3 (comprising C_{16:1}ω7c and/or C_{16:1}ω6c).

The type strain CAU 1131^T (=KCTC 52177^T=NBRC 111996^T) was isolated from reclaimed land collected from Modo in the Republic of Korea. The DNA G+C content of the type strain is 38.7 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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