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Hymenobacter rutilus sp. nov., isolated from marine sediment in the Arctic

Myong Chol Kim,[1,2] Chol Myong Kim,[1] Ok Chol Kang,[1] Yumin Zhang,[1] Zuobing Liu,[1] Danzeng Wangmu,[1] Ziyan Wei,[1] Yao Huang[1] and Fang Peng[1,*]

Abstract

Strain K2-33028^T, which appeared as a brick-red colony on an R2A plate, was isolated from a marine sediment sample from Kings Bay, Svalbard Archipelago, Norway. Phylogenetic analysis based on 16S rRNA gene sequences indicated that K2-33028^T represented a member of the genus *Hymenobacter*. Cells were Gram-reaction-negative, non-spore-forming, aerobic, rod-shaped and without motility. Growth occurred at 4–37 °C (optimum 28 °C) and at pH 6.0–8.0 (optimum pH 7.0). Cells contained menaquinone-7 as the main respiratory quinone and iso-$C_{15:0}$, summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:1}\omega5c$, summed feature 4 (comprising anteiso-$C_{17:1}$B and/or iso-$C_{17:1}$I) and anteiso-$C_{15:0}$ as the major cellular fatty acids. Phosphatidylethanolamine was predominant in the polar lipid profile. The DNA G+C content was 64.3 mol %. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain K2-33028^T is considered to represent a novel species of the genus *Hymenobacter*, for which the name *Hymenobacterrutilus*sp. nov. is proposed. The type strain is K2-33028^T (=CCTCC AB 2016091^T=KCTC 52447^T).

The genus *Hymenobacter* was first described by Hirsch *et al.* [1] and is a member of the family *Cytophagaceae* in the phylum *Bacteroidetes*. Species of the genus *Hymenobacter* are Gram-reaction-negative, rod-shaped cells that form pink to red-pigmented colonies, contain menaquinone-7 (MK-7) as the major respiratory quinone and possess iso- and anteiso-branched C_{15} fatty acids, the unsaturated fatty acid $C_{16:1}\omega5c$ and summed features 3 and 4 as the major fatty acids. Unlike other members of the family, the members of this genus show relatively high DNA G+C contents (55–65 mol%). Species of the genus have been often isolated from less extreme environment such as grass soil [2, 3], stream sediment [4], sand [5] and wetland freshwater [6]. Furthermore, some species are known to be able to survive under unfavorable conditions such as desiccation [1, 7], radiation [8, 9] and high levels of oil and heavy metals [10]. During a study on bacterial diversity in the Arctic area, strain K2-33028^T was isolated from marine sediment. This paper deals with the detailed taxonomic characterization of this isolate, which is proposed to represent a novel species of the genus *Hymenobacter*.

A bacterial strain, designated K2-33028^T, was isolated from a marine sediment (314.6 m in depth) sample in Kings Bay (78° 58′ N, 11° 50′ E), near the settlement of Ny-Ålesund in the Svalbard Archipelago, Norway. Sampling of marine sediment was carried out with clean surface-sterilized tools. The samples were kept frozen on ice during transport to the laboratory and stored at 4 °C until used. Isolation was carried out using the standard dilution plating method on R2A agar (Becton, Dickinson and Company) at 28 °C. Single colonies on these plates were purified by transferring them onto new plates and subjecting them to additional incubation for 3 days at 28 °C. The isolate was routinely cultivated on R2A agar at 28 °C and stored following lyophilization.

For 16S rRNA gene sequencing and phylogenetic analysis, genomic DNA was extracted from a fresh culture of K2-33028^T as described by Sambrook and Russell [11]. Universal bacterial primer pairs 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1540R (5′-AGAAAGGAGGTGATCCAGCC-3′) were used for amplification of the 16S rRNA gene [12]. PCR and 16S rRNA gene sequencing were performed according to the procedure described by Lin *et al.* [13]. Sequence similarity was investigated using NCBI BLAST and pairwise similarity was calculated using the EzTaxon database [14]. After multiple alignments of the data via CLUSTAL_X [15], phylogenetic trees were then reconstructed using the neighbour-

Author affiliations: [1]China Center for Type Culture Collection (CCTCC), College of Life Sciences, Wuhan University, Wuhan 430072, PR China; [2]College of Life Sciences, Kim Il Sung University, Pyongyang, DPR of Korea.
***Correspondence:** Fang Peng, fangpeng2@aliyun.com
Abbreviation: CFA, cellular fatty acids.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain K2-33028^T is KX139010.
Four supplementary figures are available with the online Supplementary Material.

joining [16], maximum-likelihood [17] and maximum-parsimony [18] algorithms in MEGA version 7.0 software [19]. Evolutionary distances for the neighbour-joining algorithm were calculated with Kimura's two-parameter method [20] and the topology of the neighbour-joining tree was evaluated by bootstrap analysis on the basis of 1000 replications [21]. Close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The root position was estimated by using the 16S rRNA gene sequence of *Escherichia coli* ATCC 11775^T (X80725) as an outgroup.

The almost-complete 16S rRNA gene sequence (1507 bp) of K2-33028^T was obtained from genomic DNA and then deposited in the GenBank database with the accession number KX139010. 16S rRNA gene sequence comparison via the EzTaxon-e Server (http://www.ezbiocloud.net/) showed that the levels of 16S rRNA gene sequence similarity between K2-33028^T and the type strains of members of the genus *Hymenobacter* (96.3–88.8 %) were below the 97.0 % threshold recommended for the discrimination of different bacterial species [22], and that K2-33028^T was most closely related to *Hymenobacter tibetensis* CCTCC AB 207089^T (96.3 % 16S rRNA gene sequence similarity) and *Hymenobacter norwichensis* DSM 15439^T (96.0 %), respectively. Results of phylogenetic analysis based on 16S rRNA gene sequences also indicated that K2-33028^T represented a member of the genus *Hymenobacter* and formed a monophyletic cluster with *H. tibetensis* CCTCC AB 207089^T and *H. norwichensis* DSM 15439^T, independent of the applied treeing method (Figs 1, S1 and S2, available in the online Supplementary Material). These results indicated that K2-33028^T can be considered to represent a novel species within the genus *Hymenobacter*. On the basis of phylogenetic data, *H. tibetensis* CCTCC AB 207089^T and *H. norwichensis* DSM 15439^T were used as the reference strains for subsequent comparisons.

Growth of the novel isolate on various standard bacteriological media was evaluated at 28 °C by using R2A agar (BD), tryptic soy broth (TSB, BD) agar, 0.1× tryptic soy broth (TSB, BD) agar, PYES agar (peptone from casein 3.0 g, yeast extract 3.0 g, sodium succinate 2.3 g, distilled water 1 l, pH 7.2, agar 15 g), Czapek's-Dox agar (sucrose 30.0 g, $NaNO_3$ 3.0 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, K_2HPO_4 1.00 g, agar 13.0 g, distilled water 1 l) and MacConkey agar (BD). Cell morphology was examined by phase-contrast (BX51; Olympus) and transmission electron (JEM-1400Plus; JEOL) microscopy using cells grown on R2A agar at 28 °C for 72 h. Gram-staining of cells was carried out as described by Doetsch [23]. Gliding motility was tested according to the procedure described by Bowman [24]. Growth at different temperatures (4, 10, 15, 20, 28, 30, 37 and 42 °C) and at different concentrations of NaCl (0, 0.5, 1, 2, 3, 4 and 5 % w/v) were investigated on R2A agar for up to 10 days. The pH range for growth was determined in R2A broth at pH 4.0–11.0 (in increments of 1.0 pH unit). Growth under anaerobic conditions was tested on R2A agar supplemented with 0.1 % KNO_3 in a GasPak (BBL) jar at 28 °C for 30 days. Oxidase activity was evaluated by the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine [25]. Catalase activity was determined by measurements of bubble production after the application of 3 % (v/v) hydrogen peroxide solution. Hydrolysis of casein was examined as described by Cowan and Steel [26] using R2A agar as the basal medium. Pigment-production was tested as described by Kämpfer *et al.* [27]: the cell pellets from cultures grown for 3 days (R2A agar) were extracted in 200 µl of an acetone: methanol mixture (7 : 2, v/v) for a few minutes. After centrifugation, the supernatant was transferred into a quartz cuvette followed by recording of absorption spectra between 300 and 900 nm in a UV/visible spectrophotometer (Power Wave XS; BioTek Instrument). Antibiotic susceptibility was tested using the disc-diffusion method [28]. After incubation at 28 °C for 3 days, strains were classifiied as sensitive or resistant, any sign of growth inhibition was scored as sensitivity to the corresponding antibiotic. Resistance to an antimicrobial drug was indicated if no inhibition zone was observed. Additional physiological and biochemical characteristics were determined by using the API 20NE and API ZYM kits and the GN2 MicroPlate (Biolog) according to the manufacturers' instructions.

In order to measure the G+C content of the chromosomal DNA from the novel strain, genomic DNA, extracted and purified according to the process described by Moore and Dowhan [29], was degraded enzymically into nucleosides and the G+C content was then determined as described by Mesbah *et al.* [30] using reversed-phase HPLC (UltiMate 3000; Dionex). Cell biomass for the analysis of respiratory quinones was obtained from cultivations on R2A broth at 28 °C for 72 h. Respiratory quinones were extracted from lyophilized cells as described by Collins *et al.* [31] and were identified by HPLC as described by Xie and Yokota [32]. For cellular fatty acid (CFA) analysis, the novel isolate and the related type strains were grown on R2A agar at 28 °C, and cells from late-exponential phase were used. Harvesting, saponification, methylation and extraction of CFAs were carried out following the protocol of the Sherlock Microbial Identification System (MIDI) version 6.0. Separation and identification of fatty acid methyl esters was performed using a 6890N gas chromatograph (Hewlett Packard), with MIDI Sherlock TSBA version 6 [33]. The polar lipids were extracted and identified using two-dimensional TLC according to the method of Tindall [34]. Polyamines were extracted and analyzed as described by Busse and Auling [35] and Busse *et al.* [36].

K2-33028^T grew well at 28 °C on R2A agar and 0.1×TSB agar, but not on TSB agar, PYES agar, Czapek's-Dox agar and MacConkey agar. Cells were strictly aerobic, Gram-reaction-negative, rods, 0.8–1.0 µm in diameter and 1.6–2.4 µm in length (Fig. S3). Motility was not observed. Colonies on R2A agar were circular, convex, smooth, shiny, brick-red and 2.0–3.0 mm in diameter within 3 days at 28 °C. Growth temperature ranged between 4 and 37 °C,

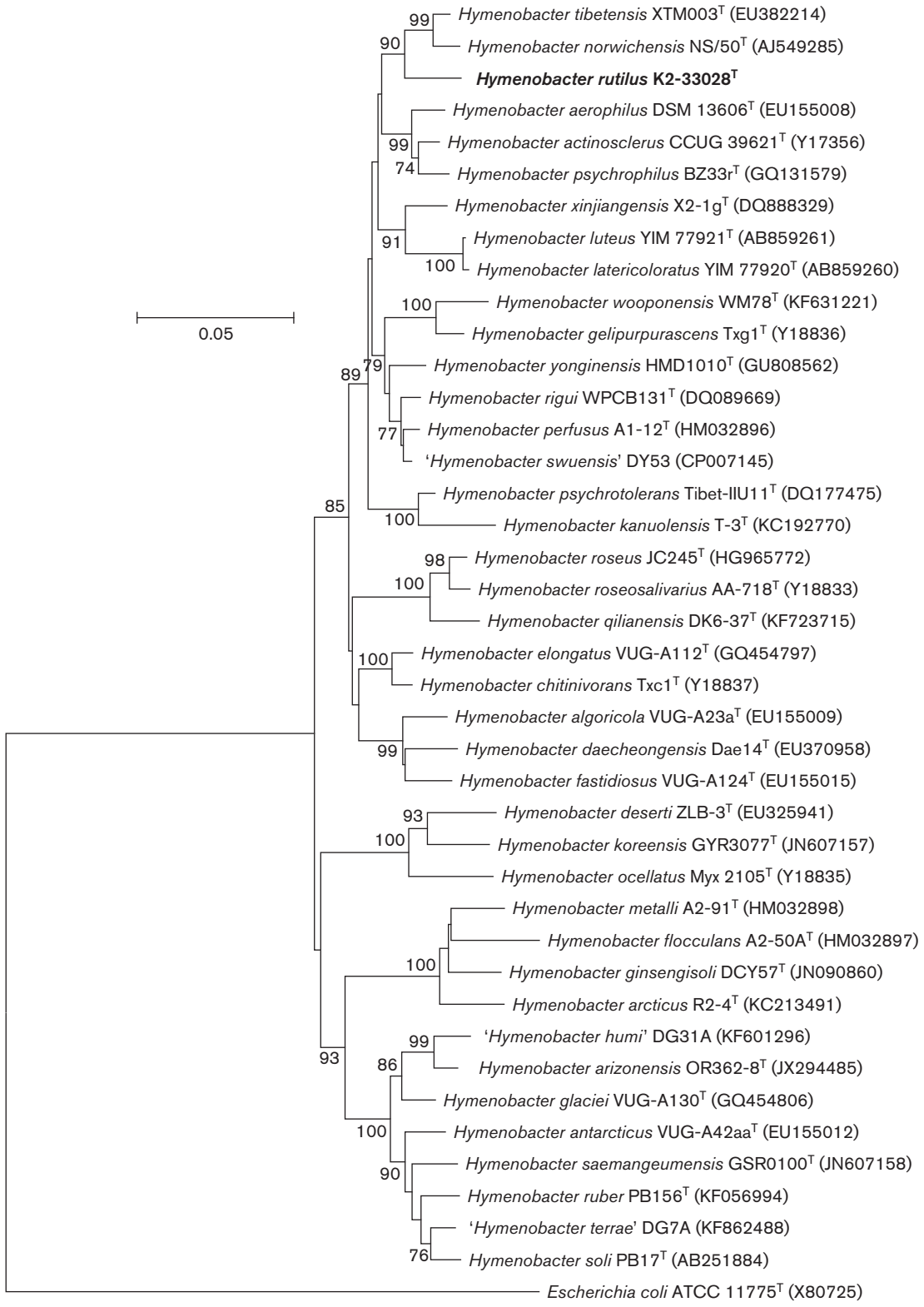

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (KX139010), showing the phylogenetic position of K2-33028^T among the members of the genus *Hymenobacter*. Bootstrap values (expressed as percentages of 1000 replications) of above 70 are shown at the branch points. The sequence of *Escherichia coli* ATCC 11775^T (X80725) was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

and growth at 42 °C was not observed. Growth occurred at pH 6.0–8.0. The optimal temperature and pH for growth were 28 °C and 7.0, respectively. The presence of more than 0.5 % (w/v) NaCl inhibited growth in R2A broth.

This isolate was positive for oxidase, catalase and hydrolysis of casein. The spectra of pigments extracted from cells of K2-33028^T and two related species showed a peak at around 480 nm with a shoulder at 505–510 nm, indicating the presence of red-series pigment in all the tested cells. The phenotypic characteristics that differentiate K2-33028^T from related species are listed in Table 1. Cells are sensitive to chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin G (10 U/IE), vancomycin (30 µg) and tetracycline (30 µg), they are resistant to polymyxin B (300 IU). Detailed results of the phenotypic and biochemical tests are given in the species description.

The fatty acid profile (Table 2) of K2-33028^T was characterized as consisting of iso-$C_{15:0}$ (20.7 %), summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 15.3 %), $C_{16:1}\omega5c$ (10.6 %), summed feature 4 (anteiso-$C_{17:1}$B and/or comprising iso-$C_{17:1}$I; 8.9 %) and anteiso-$C_{15:0}$ (7.6 %), which was similar to that of the typical species of the genus *Hymenobacter*. In addition, K2-33028^T contained higher amounts of iso-$C_{16:1}$H and iso-$C_{16:0}$ and lower proportions of iso-$C_{17:0}$, $C_{16:0}$, $C_{18:0}$ and iso-$C_{17:0}$ 3-OH when compared with the related species. The quinone system consisted of menaquinone-7 (MK-7) in accordance with the characteristics reported for other species of the genus *Hymenobacter* [1]. K2-33028^T displayed a polar lipid pattern (Fig. S4) composed of phosphatidylethanolamine (PE), five unknown aminophospholipids (APL1–5), three unknown phospholipids (PL1–3), an unknown glycolipid (GL), an unknown aminolipid (AL) and two unknown polar lipids (L1–2). In particular, the presence of phospholipids distinguished K2-33028^T from the related species. The polyamines of K2-33028^T exhibited similar patterns to that of the reference strain *H. tibetensis* CCTCC AB 207089^T, which indicated *sym*-homospermidine to be a predominant compound of polyamine in novel isolate. The DNA G+C content of strain K2-33028^T was 64.3 mol%, which is a relatively higher content compared with that of the related species.

Table 1. Characteristics that differentiate K2-33028^T from the related members of the genus *Hymenobacter*

Strains: 1, K2-33028^T; 2, *H. tibetensis* CCTCC AB 207089^T; 3, *H. norwichensis* DSM 15439^T; +, positive reaction; −, negative reaction; w; weakly positive. Data were taken from this study unless otherwise indicated. PE, phosphatidylethanolamine; APL, unknown aminophospholipid; GL, unknown glycolipid; AL, unknown aminolipid; PL, unknown phospholipid; L, unknown polar lipid.

Characteristics	1	2	3
Growth in/at			
1 % NaCl (w/v)	−	+*	−†
4 °C	+	+*	w†
37 °C	+	−*	−†
Enzyme activity (API ZYM);			
Esterase (C4)	w	+	+
Esterase lipase(C8)	+	+	w
Cysteine arylamidase	+	−	−
Trypsin	−	+	−
α-Chymotrypsin	+	−	−
Naphtol-AS-BI-phosphatase	w	+	+
α-Galactosidase	−	+	−
β-Galactosidase	−	+	+
β-Glucuronidase	w	−	−
α-Glucosidase	−	+	+
β-Glucoidase	−	+	+
N-Acetylglucosaminidase	−	+	w
Polar lipids	PE, 5 APL, 3 PL, GL, AL, 2 L	PE, 3 APL, AL, GL, 6 L*	PE, 4 APL, 2 GL, 3 L†
DNA G+C content (mol %)	64.3	55.8*	56.5†

*Data from Dai *et al.* [37].

†Data from Buczolits *et al.* [38].

Table 2. CFA composition of K2-33028^T and phylogenetically related species of the genus *Hymenobacter*

Strains: 1, K2-33028^T; 2, *H. tibetensis* CCTCC AB 207089^T; 3, *H. norwichensis* DSM 15439^T; Data was obtained from this study. Values are percentages of total fatty acids; fatty acids amounting to <1 % of the total fatty acids in all strains listed are omitted; –, Not detected; TR, traces (<1 %).

Fatty acid	1	2	3
$C_{14:0}$	TR	1.6	1.6
iso-$C_{15:1}$G	2.7	1.7	–
iso-$C_{15:0}$	20.7	25.1	20.4
anteiso-$C_{15:0}$	7.6	8.1	6.1
$C_{15:1}\omega6c$	1.4	–	–
iso-$C_{16:1}$H	4.3	–	TR
iso-$C_{16:0}$	3.6	TR	–
$C_{16:1}\omega5c$	10.6	14.1	18.5
$C_{16:0}$	4.3	6.2	12.0
iso-$C_{15:0}$ 3-OH	2.3	2.5	2.2
iso-$C_{17:0}$	TR	3.4	3.4
$C_{17:1}\omega6c$	1.3	–	–
$C_{16:0}$ 3-OH	1.5	–	–
$C_{18:1}\omega9c$	TR	2.1	–
$C_{18:0}$	3.3	5.1	6.7
iso-$C_{17:0}$ 3-OH	3.3	5.9	5.4
Summed feature 1*	1.3	TR	–
Summed feature 3*	15.3	14.8	17.5
Summed feature 4*	8.9	7.5	5.6

*Summed features are groups of two or three fatty acids that could not be separated by GLC with the MIDI System. Summed feature 1 contained iso-$C_{15:1}$H and/or $C_{13:0}$ 3-OH; Summed feature 3 contained $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; Summed feature 4 contained anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$I.

Results of phenotypic and molecular studies, especially examination of growth temperature, polar lipid pattern, CFA profile and DNA G+C content indicated that K2-33028^T can be clearly distinguished from the previously described species of the genus *Hymenobacter*. On the basis of the results from this study, K2-33028^T should be classified as a representative of a novel species of the genus *Hymenobacter*, for which the name *Hymenobacter rutilus* sp. nov. is proposed.

DESCRIPTION OF *HYMENOBACTER RUTILUS* SP. NOV.

Hymenobacter rutilus (ru′ti.lus. L. masc. adj. *rutilus* red).

Cells are Gram-reaction-negative, aerobic, non-motile, non-spore-forming and rod shaped, 0.8–1.0×1.6–2.4 μm in size. Cells grow well on R2A agar and 0.1×TSB agar but not on TSB agar, PYES agar, Czapek's-Dox agar and MacConkey agar. Growth occurs on R2A agar at 4–37 °C (optimum 28 °C) but not at 42 °C. The pH range for growth is from 6.0 to 8.0 (optimum pH 7.0). No growth occurs in the presence of NaCl. Colonies on R2A agar are circular, convex, entire, shiny, smooth, brick-red and 2.0–3.0 mm in diameter. Positive for oxidase, catalase and hydrolysis of casein. Production of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cysteine arylamidase, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase is observed. Activities of lipase (C14), trypsin, β-galactosidase, α-glucosidase, β-glucosidase, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, β-fucosidase and urease are not observed. Also negative for nitrate reduction and indole production but positive for hydrolysis of gelatin. Weakly positive for hydrolysis of aesculin. In addition, none of the carbohydrates in API 20NE are able to be used as a sole carbon source. Positive for Biolog GN2 MicroPlate substrates: L-arabinose, L-fucose, pyruvic acid methyl ester, α-ketovaleric acid, D-saccharic acid, L-glutamic acid, glycyl-L-aspartic acid, L-serine, inosine, thymidine and glycerol. The major respiratory quinone is MK-7. The major cellular fatty acids (>5 %) are iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:1}$ $\omega5c$, summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}$ $\omega6c$) and summed feature 4 (anteiso-$C_{17:1}$B and/or iso-$C_{17:1}$I). The polar lipid pattern is composed of the major compound phosphatidylethanolamine and five unknown aminophospholipids, three unknown phospholipids, an unknown glycolipid, an unknown aminolipid and two unknown polar lipids. *sym*-Homospermidine is the predominant compound in the polyamine pattern.

The type strain, K2-33028^T (=CCTCC AB 2016091^T=KCTC 52447^T), was isolated from marine sediment sample from Kings Bay, near Ny-Ålesund, Svalbard Archipelago, Norway. The DNA G+C content of the type strain is 64.3 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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