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NOTE

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Luteolibacter flavescens sp. nov., isolated from deep seawater

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Abstract

A novel Gram-stain-negative, rod-shaped, non-motile strain, designated GKX^T, was isolated from deep seawater. Strain GKX^T was able to grow at 20–35 °C (optimum, 25 °C), pH 5.5–9.5 (optimum, 7.5) and 0–4.0 % (w/v) NaCl (optimum, 1.0 %). The major fatty acids were $C_{16:1}\omega9c$ (15.4 %), $C_{16:0}$ (18.4 %), $C_{14:0}$ (12.0 %), iso-$C_{14:0}$ (30.1 %) and anteiso-$C_{15:0}$ (5.7 %). Strain GKX^T contained phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid as the main polar lipids. The only isoprenoid quinone was menaquinone-9. The diagnostic amino acids of the cell-wall peptidoglycan contained *meso*-diaminopimelic acid. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain GKX^T belonged to the genus *Luteolibacter* in the family *Verrucomicrobiaceae*. The 16S rRNA gene sequence of this strain showed 98.0, 93.5 and 93.3 % sequence similarity, respectively, with those of *Luteolibacter arcticus* MC 3726^T, *L.uteolibacter pohnpeiensis*A4T-83^T and *L.uteolibacter cuticulihirudinis* E100^T. DNA–DNA hybridization value of strain GKX^T with *L. arcticus* MC 3726^T was 33.1 %. The G+C content of the genomic DNA was 59.5 mol%. On the basis of the genotypic, phenotypic, phylogenetic and chemotaxonomic characteristics, strain GKX^T was proposed to represent a novel species of the genus *Luteolibacter*, named *Luteolibacter flavescens* sp. nov. (type strain GKX^T=MCCC 1K03193^T=KCTC 52361^T).

The genus *Luteolibacter*, belonging to the family *Verrucomicrobiaceae* within the phylum *Verrucomicrobia*, currently comprises six species: *Luteolibacter pohnpeiensis*, *L. algae* [1], *L. luojiensis* [2], *L. cuticulihirudinis* [3], *L. yonseiensis* [4] and *L. arcticus* [5]. The type strains of the recognized species in this genus are all Gram-negative, non-motile and coccoid- or rod shaped. The activity of nitrate reduction is negative in most of the type strains in the genus *Luteolibacter*. The major respiratory quinone is menaquinone-9 (MK-9), while some species contain MK-8, MK-7 or MK-10 as well. Predominant cellular fatty acids are $C_{16:0}$ and iso-$C_{14:0}$. The DNA G+C contents of type strains vary between 47.2 and 60.7 mol%. In this study, we present a novel strain named GKX^T, which was isolated from deep seawater in the South China Sea and identified as a novel species of the genus *Luteolibacter* based on phylogenetic, genomic, chemotaxonomic and phenotypic characteristics.

A bacterial strain designated GKX^T was isolated from a seawater sample, which was collected in October 2011 from the South China Sea (19° 22′ N 115° 38′ E) at the depth of 2.5 km. The sample was stored at 4 °C in the lab until it was used. The seawater sample was serially diluted with a sterile 0.85 % (w/v) NaCl solution and plated onto a modified Gause synthetic agar plate (soluble starch 10 g, KNO_3 1 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, agar 15 g, deionized water 1000 ml, pH 7.4) at 28 °C according to the standard dilution plating method [6] for isolation. Single colonies on these plates were purified by transferring them onto new plates and subjecting them to an additional incubation at 28 °C for 72 h. For long-time storage, the strain was preserved as suspensions with 30 % (v/v) glycerol at −80 °C and was freeze-dried, then stored at −20 °C as well.

Growth was evaluated at 28 °C on several standard bacteriological media: R2A agar, 0.3× R2A agar, tryptic soy broth (TSB; BD) agar, 0.1× TSB (BD) agar, marine broth 2216 (BD) agar, 0.1× marine broth 2216 (BD) agar, nutrient agar (NA), potato dextrose agar and MacConkey agar (BD). Gram-staining reaction was performed according to Claus [7]. The presence of flagella and cell morphology were observed by transmission electron microscopy (JEM-1230; JEOL) when cells were in the exponential phase of growth on the modified Gause synthetic agar plate at 28 °C. The presence of gliding motility was checked by the hanging drop method as described by Suzuki *et al.* [8]. The

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Abbreviation: MK-9, menaquinone-9.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GKX^T is KX185934.
One supplementary figure is available with the online Supplementary Material.

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temperature range for growth was determined in the modified Gause synthetic broth at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40 and 45 °C for up to 2 weeks. The pH range for growth in the modified Gause synthetic broth was measured from 5.0 to 10.0 with an interval of 0.5 pH. Forty millimolar MES (pH 5.0–6.0), PIPES (pH 6.5–7.5), Tricine (pH 8.0–8.5) and CAPSO (3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid) (pH 9.0–10.0) were used as pH buffers, respectively. Salt tolerance was tested with the concentrations of NaCl at 0, 0.5, 1, 1.5, 2, 3, 4, 5 and 10 % (w/v) in the modified Gause synthetic broth for 2 weeks at 28 °C. Anaerobic growth was determined with microaerobic system (Anaero-Pack-MicroAero, 2.5 litres, Mitsubishi Gas Chemical) using the modified Gause synthetic agar, to which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate were added as electron acceptors [9]. Pigment analysis was performed as described by Kim *et al.* [5] and the absorption spectrum was measured at 300–800 nm [10] with acetone/methanol mixture (7 : 2, v/v) as a blank.

Catalase and oxidase activities were tested by the methods as described by Wu *et al.* [11] and Kovacs [12], respectively. Nitrate reduction and hydrolysis of CM-cellulose, filter paper, casein and gelatin were determined according to Sun *et al.* [13]. H_2S production, indole production, methyl red and Voges–Proskauer tests were assayed according to Zhang *et al.* [14]. Degradation of starch, L-tyrosine and hydrolysis of Tweens 20, 40, 60 and 80 were examined as described by Sun *et al.* [15]. Hydrolysis of hypoxanthine and xanthine was tested as described by Gordon and Mihm [16]. Other biochemical properties and enzyme activities were tested using API ZYM, API 20NE (BioMérieux) and the GN2 MicroPlate (Biolog) according to the manufacturers' instructions.

For 16S rRNA gene sequencing and phylogenetic analysis, the genomic DNA of the strain GKX^T was extracted by using a Quick Bacteria Genomic DNA Extraction kit (Dongsheng Biotech). PCR was performed using universal primer pair 27F (5′-GAGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGAC-3′) to amplify the 16S rRNA gene. The purified PCR products were cloned into the vector pMD19-T (Takara) and then sequenced. The sequence was uploaded and compared with closely related species with EzTaxon server [17]. Multiple sequences alignment was performed with CLUSTAL W 1.8 program of the MEGA5 package [18, 19]. A total of 1520 nt in unambiguously aligned positions were used for tree reconstruction. Phylogenetic trees were reconstructed using the following three methods: the neighbour-joining [20], maximum-likelihood [21] and maximum-parsimony [22] methods with the MEGA5. Evolutionary distances used in the neighbour-joining method were calculated according to the algorithm of the Kimura two-parameter model [23]. Bootstrap analysis was based on 1000 replications. The G+C content of the genomic DNA was determined by HPLC according to Mesbah *et al.* [24]. DNA–DNA hybridization values of strain GKX^T and *L. arcticus* MC 3726^T were determined by using a Beckman DU800 spectrophotometer according to the method of Zhang *et al.* [25]. Hybridization temperature was set at 57 °C.

For the analysis of respiratory quinones and polar lipids, cell biomass was obtained when cells were at late-exponential phase and lyophilized. Respiratory quinones were extracted as described by Collins *et al.* [26] and identified by HPLC as described by Minnikin *et al.* [27]. The polar lipids were extracted by a mixed solution of chloroform/methanol/water (1 : 2 : 1, by vol.), separated on silica gel 60 F254 plate (10×10 cm, Merck) and the results were further analysed as described by Xu *et al.* [28]. Fatty acid methyl esters were analysed according to the instructions of the Microbial Identification System (MIDI, Microbial ID) with the standard MIS library Generation Software version 4.5. For fatty acid methyl esters analysis, strain GKX^T and the reference strains were grown on R2A agar at 28 °C until cells were at late-exponential phase. Cell-wall peptidoglycan was prepared and hydrolysed by the methods of Kawamoto *et al.* [29] and the amino acid composition was analysed with an automatic amino acid analyser (Hitachi L-8900) and HPLC.

Strain GKX^T grew well at 28 °C on R2A agar, 0.3× R2A agar, NA and 0.1× TSB agar, but not on TSB agar, marine broth 2216 agar, 0.1× marine broth 2216 agar, potato dextrose agar or MacConkey agar. However, *L. pohnpeiensis* grew well on 0.1× marine broth 2216 agar and *L. arcticus* did not grow on either NA or 0.1× TSB agar [5]. The cells were aerobic, Gram-stain-negative and rod shaped. No gliding motility was observed. Colonies on the modified Gause synthetic agar plate were circular, slightly convex, smooth, yellow in colour and approximately 2 mm in diameter after incubation for 3 days at 28 °C. No flagella were observed under transmission electron microscopy. The pH range for growth was pH 5.5–9.5. For temperature range, growth occurs between 20 and 35 °C. No growth was monitored at either 4 or 37 °C after 14 days. Optimal growth occurred at pH 7.5 and 25 °C. The NaCl concentration range for growth was 0–4.0 % (w/v) with an optimum of 1.0 %. For *L. cuticulihirudinis* and *L. arcticus*, no growth occurred at or above 1.0 % NaCl concentration, while *L. pohnpeiensis* showed no growth at 0 % NaCl concentration [1, 3, 5]. The pigments extracted from cells of strain GKX^T showed UV–visible spectra with absorption maxima at 446 and 474 nm, characteristic of carotenoids.

The PCR-amplified 16S rRNA gene sequence of strain GKX^T comprised 1482 bp. The result of sequence similarity analysis based on the 16S rRNA gene sequence showed 98.0, 93.5 and 93.3 % sequence similarity, to those of the type strains of *L. arcticus* MC 3726^T, *L. pohnpeiensis* A4T-83^T and *L. cuticulihirudinis* E100^T, respectively. In the phylogenetic tree reconstructed using the maximum-likelihood algorithm, strain GKX^T was placed within the monophyletic cluster of the genus *Luteolibacter*, and strain GKX^T was in the subcluster comprising the type strain of *L. arcticus* by a bootstrap resampling value of 100 % (Fig. 1). In addition, with the almost

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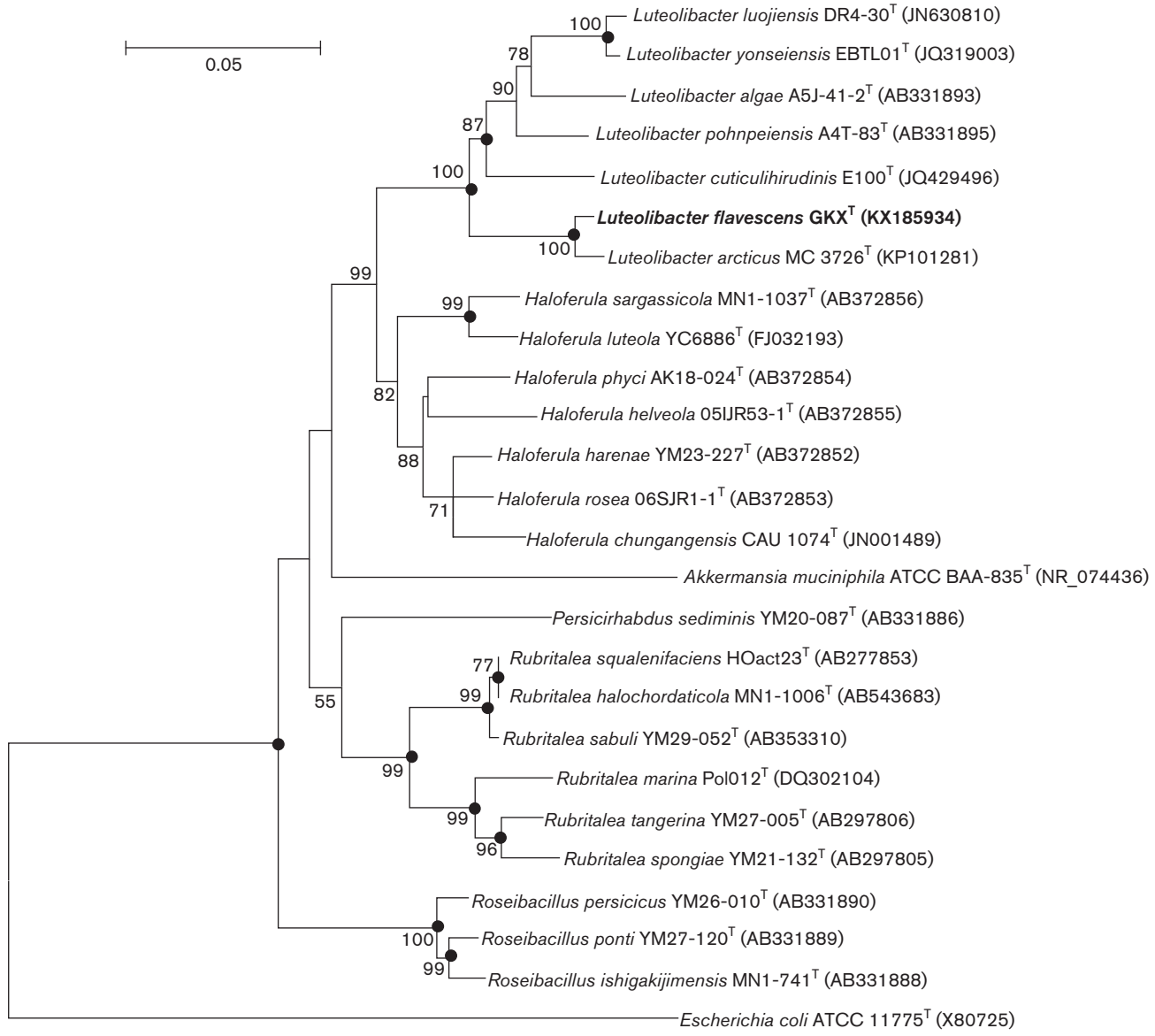

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the relationship of strain GKX^T with the related taxa. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bootstrap values were based on 1000 replications. Bar, 0.05 substitutions per nucleotide position. Only values above 50 % are shown. Filled circles indicate nodes also obtained in both neighbour-joining and maximum-parsimony trees.

same topological structure, the neighbour-joining and maximum-parsimony trees also showed the closer phylogenetic relationship of strain GKX^T and type strains of *L. arcticus*, *L. pohnpeiensis* and *L. cuticulihirudinis* (Fig. 1). Among the genus *Luteolibacter*, *L. arcticus* MC 3726^T, *L. pohnpeiensis* A4T-83^T (the type strain of the genus *Luteolibacter*) and *L. cuticulihirudinis* E100^T were selected as the reference strains for direct comparison to our novel strain.

As for the chemotaxonomic characteristics, strain GKX^T had some features different from its three closest neighbours in the genus *Luteolibacter*. Strain GKX^T contained only one type of isoprenoid quinone, MK-9. But all the three reference strains contained one or more types of isoprenoid quinone in addition to MK-9. The complete fatty acid profile of strain GKX^T was compared with those of the type strains of *L. arcticus*, *L. pohnpeiensis* and *L. cuticulihirudinis* in Table 1. The major fatty acids (>5 % of the total fatty acids) found in strain GKX^T were $C_{16:1}\omega 9c$ (15.4 %), $C_{16:0}$ (18.4 %), $C_{14:0}$ (12.0 %), iso-$C_{14:0}$ (30.1 %) and anteiso-$C_{15:0}$ (5.7 %). The proportion of $C_{14:0}$, iso-$C_{14:0}$ and anteiso-$C_{15:0}$ fatty acids was similar to the other three reference strains of genus *Luteolibacter*, though proportional differences existed. Most of the content of fatty acids in strain GKX^T was between that

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Table 1. The cellular fatty acid contents of strain GKX^T and type strains of phylogenetically related species

Strains: 1, GKX^T; 2, *L. pohnpeiensis* A4T-83^T; 3, *L. cuticulihirudinis* E100^T; 4, *L. arcticus* MC 3726^T. Data were obtained in this study. Values are the percentage 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	4
Straight-chain				
$C_{14:0}$	12.0	15.5	6.0	10.1
$C_{15:0}$	2.1	1.0	TR	TR
$C_{16:0}$	18.4	25.4	28.1	12.9
Unsaturated				
$C_{16:1}\omega9c$	15.4	–	10.9	23.9
$C_{18:1}\omega9c$	1.4	–	–	TR
Branched				
iso-$C_{14:0}$	30.1	31.4	39.3	37.3
anteiso-$C_{15:0}$	5.7	4.8	3.0	3.8
iso-$C_{16:1}$	TR	1.2	5.6	1.1
Hydroxy				
iso-$C_{14:0}$ 3-OH	1.5	–	TR	4.6
$C_{16:0}$ 3-OH	3.0	TR	TR	TR
$C_{13:1}$ AT 12–13	TR	TR	1.3	TR
Summed feature*				
2	2.0	1.5	1.1	TR
3	–	12.8	–	–
5	4.3	–	–	TR

*Summed features are groups of two or three fatty acids that could not be separated by GLC with the MIDI System. Summed feature 2 contains $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$; summed feature 3 contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; summed feature 5 contained $C_{18:2}$ $\omega6,7c$ and/or anteiso-$C_{18:0}$.

of the other three type strains, even though some differences in the proportions of some fatty acids existed. Summed feature 5 ($C_{18:2}\omega6,7c$ and/or anteiso-$C_{18:0}$) was found in strain GKX^T with a relatively high ratio of 4.3 %, but not in *L. pohnpeiensis* or *L. cuticulihirudinis*, and found in the type strain of *L. arcticus* with a low ratio (<1 %), which was a particular feature distinguishing strain GKX^T from other three recognized species. Strain GKX^T contained phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unknown glycolipid as the major polar lipids, which was similar to that of *L. arcticus*, *L. pohnpeiensis* and *L. cuticulihirudinis* (Fig. S1, available in the online Supplementary Material). Strain GKX^T also contained four unidentified phospholipids (PL1, PL2, PL3, PL4) as minor components, which distinguished strain GKX^T from other related species (Fig. S1). The diagnostic amino acid in the cell-wall peptidoglycan of strain GKX^T was *meso*-diaminopimelic acid.

The DNA G+C content of strain GKX^T was 59.5 mol% (HPLC). The DNA–DNA hybridization value of strain GKX^T and *L. arcticus* MC 3726^T was 33.1 %, which was below the threshold value (70 %) for determining bacterial species [30].

Physiological and biochemical characteristics that differentiate strain GKX^T from its related species in the genus *Luteolibacter* are shown in Table 2. Strain GKX^T showed catalase- and oxidase-positive activities. It reduced nitrate to nitrite, whereas the type strains of *L. arcticus*, *L. pohnpeiensis* and *L. cuticulihirudinis* did not reduce nitrate to nitrite. Strain GKX^T and type strain of *L. arcticus* contained some similar characteristics in the hydrolysis of starch, methyl red test and utilization of urea, but not in the hydrolysis of skimmed milk, hydrolysis of Tweens 40, 60 and 80, assimilation of glucose and *N*-acetylglucosamine and activities of valine arylamidase, *N*-acetyl-β-glucosaminidase and α-mannosidase. Strain GKX^T did not utilize L-fucose, L-rhamnose and D-galactose as sole carbon sources, which was different from the type strains of *L. arcticus*, *L. pohnpeiensis* and *L. cuticulihirudinis*.

On the basis of the phylogenetic, genomic, chemotaxonomic and phenotypic characteristics, we propose that strain GKX^T represents a novel species of the genus *Luteolibacter*, for which the name *Luteolibacter flavescens* sp. nov. is proposed.

DESCRIPTION OF *LUTEOLIBACTER FLAVESCENS* SP. NOV.

Luteolibacter flavescens (fla.ves′cens. L. masc. adj. *flavescens* yellowish).

Cells are Gram-strain-negative, non-flagellated, non-gliding and rod shaped (0.5–0.7×0.9–1.5 μm). Anaerobic growth is not observed. After growing on a modified Gause synthetic agar plate for 3 days at 28 °C, colonies are circular, slightly convex, smooth, yellow and approximately 2 mm in diameter. The pH range for growth is pH 5.5–9.5. Growth occurs between 20 and 35 °C, but not at 37 or 4 °C. Optimal growth occurs at pH 7.5 and 25 °C. The NaCl concentration range for growth is 0–4.0 % (w/v) with an optimum of 1.0 %. Cells grow well at 28 °C on R2A agar, 0.3× R2A agar, NA and 0.1× TSB agar, but not on TSB agar, marine broth 2216 agar, 0.1× marine broth 2216 agar, potato dextrose agar or MacConkey agar. H_2S is produced. Carotenoids are produced. Catalase- and oxidase activities are positive. Nitrate can be reduced to nitrite, but nitrite cannot be further reduced into nitrogen. Indole is not produced. Methyl red and Voges–Proskauer tests are negative. Starch, Tweens 40, 60, 80 and skimmed milk are hydrolysed, but casein, CM-cellulose, filter paper, hypoxanthine, Tween 20, gelatin, L-tyrosine and xanthine are not. In the API ZYM test, alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-galactosidase activities are present. Esterase (C4), esterase lipase (C8), valine arylamidase and α-glucosidase activities are weakly positive, while lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. In assays with the API 20NE system, positive results were found in reduction of nitrate, hydrolysis of urea, aesculin, β-galactosidase, assimilation of glucose, *N*-acetylglucosamine and maltose, weakly positive results in

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Table 2. Characteristics that differentiate strain GKX^T from related members of the genus *Luteolibacter*

Strains: 1, GKX^T; 2, *L. pohnpeiensis* A4T-83^T; 3, *L. cuticulihirudinis* E100^T; 4, *L. arcticus* MC 3726^T; +, positive reaction; −, negative reaction; w, weakly positive reaction; DPG, diphosphatidylglycerol; GL, glycolipid; *meso*-DPA, *meso*-diaminopimelic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmethylethanolamine; APL, aminophospholipid; PL, unknown phospholipid; L, unknown lipid; AGL, aminoglycolipid; PGL, phosphoglycolipid.

Characteristic	1	2	3	4
Isolation source	Water of the South China Sea	Driftwood[a]	Skin of the medical leech[b]	Arctic tundra soil[c]
Catalase	+	+	−	+
Oxidase	+	+	+	+
Nitrate reduction	+	−	−	−
Methyl red	−	+	−	−
Voges–Proskauer	−	−	−	w
Growth range				
Temperature (°C)	20–35	20–37[a]	15–30[b]	4–37[c]
pH	5.5–9.5	6.5–9.0[a]	6.5–8.5[b]	5.0–9.0[c]
NaCl (w/v)	0–4.0 %	≤6 % (not at 0%)[a]	0% (not at 1%)[b]	0% (not at 1%)[c]
Hydrolysis of				
Starch	+	−	−	+
Skimmed milk	+	+	−	−
Tween 40	+	+	+	−
Tween 60	+	+	+	−
Tween 80	+	−	−	−
Urea	+	−	−	+
Assimilation of (API 20NE)				
Glucose	+	w	+	−
N-Acetylglucosamine	+	−	+	−
Utilization of				
L-Fucose	−	+	+	+
D-Galactose	−	+	+	+
Methyl β-D-glucoside	+	+	−	−
L-Rhamnose	−	+	+	+
D-Glucuronic acid	+	−	−	+
Glucuronamide	+	+	−	−
Enzyme activity (API ZYM)				
Valine arylamidase	w	−	w	−
Trypsin	−	+	w	−
α-Galactosidase	−	w	+	−
β-Glucosidase	−	w	+	−
N-Acetyl-β-glucosaminidase	−	+	+	+
α-Mannosidase	−	w	−	+
Polar lipid	PE, PME, PG, DPG, GL, PL1–4	PE, PG, DPG, GL, APL	PE, PG, DPG, GL, L1–2, PL, APL	PE, PME, PG, DPG, GL, AGL, PGL
Quinone	MK-9	MK-9, -10[a]	MK-7, -8, -9[b]	MK-9, -10[c]
Cell-wall peptidoglycan	*meso*-DPA	*meso*-DPA[a]	*meso*-DPA[c]	*meso*-DPA[c]
DNA G+C content (mol%)	59.5	55.8[a]	47.2[c]	60.7[c]

Data without symbols are all from this study.

[a]Data from Yoon *et al.* [1].

[b]Data from Glaeser *et al.* [3].

[c]Data from Kim *et al.* [5].

assimilation of arabinose, mannose and mannitol, and negative results in production of indole, fermentation of glucose, arginine dihydrolase, hydrolysis of gelatin (bovine origin), assimilation of potassium gluconate, caprate, adipate, malate, citrate and phenylacetate. The following compounds are utilized as sole carbon sources (Biolog GN2 MicroPlate):

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dextrin, Tween 40, Tween 80, L-arabinose, cellobiose, D-fructose, gentiobiose, α-D-glucose, lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucoside, raffinose, D-sorbitol, sucrose, trehalose, turanose, pyruvic acid methyl ester, D-galacturonic acid, D-glucuronic acid and glucuronamide. The predominant isoprenoid quinone is MK-9. The major fatty acids (>5 % of the total fatty acids) are $C_{16:1}\omega 9c$, $C_{16:0}$, $C_{14:0}$, iso-$C_{14:0}$ and anteiso-$C_{15:0}$. Phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, four unidentified phospholipids (PL1, PL2, PL3, PL4) and an unidentified glycolipid are the total polar lipids. The diagnostic amino acid in the cell-wall peptidoglycan is *meso*-diaminopimelic acid.

The type strain, GKX^T (=MCCC 1K03193^T=KCTC 52361^T), was isolated from deep seawater in the South China Sea. The DNA G+C content of the type strain is 59.5 mol%.

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

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