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ORIGINAL PAPER

Glaciibacter flavus sp. nov., isolated from a lichen sample

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Abstract

A novel Actinobacterium strain YIM 131861 ^{T}, was isolated from lichen collected from the South Bank Forest of the Baltic Sea, Germany. It was Gram-stain-positive, strictly aerobic, catalase positive and oxidase negative, yellow pigmented. Cells were motile with a polar flagellum, irregular rod shaped and did not display spore formation. The strain grew at 15 – 30 °C (optimum 25 °C), at pH 6.0 – 10.0 (optimum pH 7.0) and in the presence of 0 – 1.5% (w/v) NaCl (optimum 1%). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM 131861 ^{T} belonged to the genus *Glaciibacter*, and exhibited a high sequence similarity (96.4%) with *Glaciibacter superstes* NBRC 104264 ^{T}. The genomic DNA G + C content of strain YIM 131861 ^{T} was 68.2 mol%. The average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values between strain YIM 131861 ^{T} and *Glaciibacter superstes* NBRC 104264 ^{T} were 73.2 and 19.9% based on the draft genome sequence. The cell-wall peptidoglycan type was B2γ and contained the 2, 4-diaminobutyric acid as the diagnostic amino acid. Whole cell sugars were galactose, rhamnose, ribose and glucose. It contained MK-12 and MK-13 as the predominant menaquinones. The major cellular fatty acids (> 10%) were identified as *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$ and *anteiso*-$C_{17:0}$. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol and two unknown glycolipids. Based on the results of phenotypic, chemotaxonomic and phylogenetic analyses, strain YIM 131861 ^{T} should belong to the genus *Glaciibacter* and represents a novel species of the genus *Glaciibacter*, for which the name *Glaciibacter flavus* sp. nov. is proposed. The type strain is YIM 131861 ^{T} (= CGMCC 1.16588 ^{T} = NBRC 113572 ^{T}).

Keywords *Glaciibacter* · *Glaciibacter flavus* sp. nov. · Lichen

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Abbreviations

R2A	Reasoner's 2A
TSA	Tryptic soy agar
TSB	Tryptic soy broth
ANI	Average Nucleotide Identity
dDDH	Digital DNA–DNA hybridization
DPG	Diphosphatidylglycerol
PG	Phosphatidylglycerol
GL	Glycolipid

Introduction

The genus *Glaciibacter* was first described by Katayama (2009), belonging to the family *Microbacteriaceae*, and comprises a single species as to August 2020, *Glaciibacter superstes*. Lichens are structured associations of a fungus with a cyanobacteria and/or green algae in a symbiotic relationship, which provide specific habitats for diverse bacterial communities, including actinomycetes (Liu et al.

2017). To investigate more species of culturable microorganisms in lichen samples, a novel actinobacterium strain YIM 131861 ^{T} was isolated from lichen samples collected from the South Bank Forest of the Baltic Sea in Germany, and the strain was studied by a polyphasic taxonomic approach.

Methods and materials

Strain and culture conditions

Lichens are structured associations of a fungus with a cyanobacteria and/or green algae in a symbiotic relationship, which provide specific habitats for diverse bacterial communities, including actinomycetes. Lichens also provide an extremely rich reservoir for the isolation of novel species. Strain YIM 131861 ^{T} was isolated during the course of an investigation on diversity of cultivable actinobacteria in lichen symbiotic system from *Candelaria* sp. The lichen *Candelaria* sp. sample was collected from the South Bank Forest of the Baltic Sea (10°12′E, 54°31′N), Germany, and transferred to sterile paper bag immediately and air-dried in a drying oven at 28 °C for 7 days. The lichen sample was pretreated through two steps: a 5-min wash with running water, followed by three washes in sterile water. Finally, the lichen sample was homogenised with 18 ml of sterile 0.1% pyrophosphate using a sterile glass homogeniser. Strain YIM 131861 ^{T} was isolated using a standard dilution plate method on humic acid-vitamin (HV) agar (humic acid 1.0 g, Na_2HPO_4 0.5 g, KCl 1.7 g, $MgSO_4{\cdot}7H_2O$ 0.05 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, $CaCO_3$ 0.02 g, agar 18 g, B-vitamins 3.7 mg, pH 7.2) (Hayakawa et al. 1987). The isolated colony was selected and further purified on Reasoner's 2A (R2A; MB cell). Strain YIM 131861 ^{T} was cultured routinely on R2A agar plates and was preserved in 20% (v/v) glycerol suspensions at -80 °C. The isolation procedure was performed as described by Liu et al. (2017). The reference strain, *Glaciibacter superstes* NBRC 104264 ^{T} was obtained from NITE Biological Resource Center (NBRC), Japan.

Morphological, cultural, physiological and biochemical characterisations

Morphological characteristics were observed using a scanning electron microscope (ESEM-TMP, Philips), and the flagella of the strain was observed via transmission electron microscopy (JEM-2100, JEOL) after cells grown on R2A agar at 25 °C for 5 days. Cell motility determination was carried out by observing the development of turbidity throughout a tube using R2A semisolid medium containing 0.4% agar (Skerman 1967). Gram-staining test was performed using a bioMérieux Gram-stain kit according to the manufacturer's instructions and the Schaeffer-Fulton method, respectively (Gregersen 1978 and Smibert et al. 1981). Anaerobic growth was determined after incubation on R2A for 14 days at 25 °C using the GasPak EZ Anaerobe Pouch System (BD). Growth at various temperatures in the range − 5 to 45 °C (-5, 0, 4, 10, 15, 20, 25, 28, 30, 37, 40 and 45 °C) was determined on R2A for 5 days. The pH range for growth (pH 4.0–13.0 at intervals of 1.0 pH unit) was tested in tryptic soy broth (TSB; Difco) at 25 °C using the buffer system described by Xu et al. (2005). Growth with added salts was tested on R2A agar containing 0–7% NaCl (w/v, at 0.5% intervals). Oxidase activity was observed by oxidation of tetramethyl-phenylenediamine. Catalase activity was determined by production of bubbles after the addition of a drop of 3% H_2O_2. Urease activity, milk coagulation and peptonization, H_2S production, nitrate reduction, gelatine liquefaction, hydrolysis of starch, cellulose and Tweens (20, 40, 60 and 80) were tested as described by Smibert and Krieg (1994). The biochemical properties of strain YIM 131861 ^{T} were determined using API ZYM, API 20NE and API 50CH kits (bioMérieux) according to the manufacturer's protocols.

16S rRNA gene sequencing and phylogenetic analyses

Extraction of genomic DNA sequence and PCR amplification of the 16S rRNA gene were done as described by Li et al. (2007). The 16S rRNA gene sequence obtained in this study was compared with sequences from EzBioCloud using blast (https://www.ezbiocloud.net/) (Yoon et al. 2017a, b). Multiple alignments with corresponding sequences of the closely related strains were aligned using CLUSTAL X 1.83 (Thompson et al. 1997). Phylogenetic analysis were performed using the software package MEGA version 7.0 (Kumar et al. 2016). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou et al. 1987), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) tree-making algorithms. The method used to compute evolutionary distances was Kimura's two-parameter model (Kimura 1980). Bootstrap values were calculated based on 1000 replications (Felsenstein 1985).

Genomic analyses

The whole-genome sequence was determined using the Illumina HiSeq 4000 sequencing platform. The draft genome was assembled using SOAP denovo version 2.04 and the short oligonucleotide of assembled result was further polished using SOAP aligner 2.21, respectively (Li et al. 2008 and Li et al. 2015). The average nucleotide identity (ANI) was calculated using the orthoANIu algorithm on EzBioCloud (Lee et al. 2016) and ANI calculator (Yoon et al. 2017a, b). The estimated genome-sequence based digital DNA–DNA hybridization (dDDH) values were calculated using formula 2 at the

Genome-to-Genome Calculator (CGGC) website (https://ggdc.dsmz.de/ggdc.php) as described by Meier-Kolthoff et al. (2013). Genes annotation were conducted through the NCBI prokaryotic genome annotation pipeline.

Chemotaxonomic characterisation

Cell mass of YIM 131861 ^{T} and reference strain for chemical analysis was harvested after incubation at 25 °C for 5 days on R2A medium except for fatty acid analysis for which the cells were grown on tryptic soy agar (TSA, Difco) plates for 5 days at 25 °C. Polar lipids were extracted and analysed as described by Minnikin et al. (1984) using two dimensional TLC (silica gel 60 plates; Merck). Menaquinones were extracted by the method of Collins et al. (1977) and detected by HPLC (Tamaoka et al. 1983). Cellular fatty acid analysis was performed using the Sherlock Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6) (Sasser 1990) according to the manufacturer's instructions. Cell wall amino acids and whole-cell sugars were extracted, detected and analysed according to procedures described by Schleifer and Kandler (1972) and Tang et al. (2009).

Results and discussion

Morphological, cultural, physiological and biochemical characterisations

Cells of strain YIM 131861 ^{T} were strictly aerobic, Gram-stain-positive, no-spore-forming and motile with a polar flagellum. The cells were observed as straight or curved rod-shaped, $0.4-0.5\times1.0-1.6$ μm in size, and circular, smooth and glistening yellow colonies were formed on R2A medium. The strain YIM 131861 ^{T} was negative for oxidase and positive for catalase. Urease activity, milk coagulation and peptonization, H_2S production, gelatine liquefaction and hydrolysis of cellulose and Tween 80 were negative. The strain showed the following enzyme activities: positive for esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase; and other tests were negative in the API ZYM system (bioMérieux). In the API 20NE tests, hydrolysis of aesculin and PNPG, and assimilation of D-glucose were positive. Differential phenotypic characteristics between strain YIM 131861 ^{T} and Glaciibacter superstes NBRC 104264 ^{T} are shown in Table 1.

Table 1 Phenotypic characteristics that differentiate strain YIM 131861 ^{T} from closely related species

Characteristic	1	2
Isolation source	Lichen	Permafrost ice wedge
Colony colour	Yellow	White
Growth conditions		
Temperature (°C)	15–30	− 5–25
pH	6.0–10.0	5.0–12.0
NaCl concentration (%, w/v)	0–1.5	0–4
Nitrate reduction	−	+
Hydrolysis of		
Tweens 20, 40, 60	−	+
Starch	+	−
Enzyme activity		
Esterase (C4)	+	w
Trypsin	−	+
α-galactosidase	w	+
β-galactosidase	w	+
β-glucosidase	+	−
N-acetyl-β-glucosaminidase	+	−
α-mannosidase	−	+
Acid produced from		
D-ribose	−	+
L-xylose	−	+
Esculine citrate	+	−
D-maltose	−	+
Xylitol	−	+
Glycogene	+	−
D-turanose	−	+
Major polar lipids	DPG, PG, GL_{1-2}	DPG, PG, GL_{1-3}
DNA G + C content (mol%)	68.2	65.0*

Strains: 1, YIM 131861 ^{T}; 2, *Glaciibacter superstes* NBRC 104264 ^{T}. +, Positive; −, negative; w, weak. All data were obtained from this study except where indicated. Both strains were motile by means of a polar flagellum. Urease activity, milk coagulation and peptonization, H_2S production, gelatine liquefaction and hydrolysis of cellulose and Tweens 80 were negative in both strains. In API 20NE tests, all strains were positive for hydrolysis of aesculin and PNPG. In the API ZYM kits, all strains were positive for esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. In the API 50CH kits, all strains were positive for acid production from glycerol, L-arabinose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol and D-cellobiose

*Data from Katayama et al. (2009)

16S rRNA gene sequencing and phylogenetic analyses

The complete available 16S rRNA gene sequence of strain YIM 131861 ^{T} was 1566 bp (GenBank accession number MK608324). Based on the 16S rRNA gene sequence comparisons, strain YIM 131861 ^{T} was found to have the highest level of similarity with *Glaciibacter superstes* NBRC 104264 ^{T} (96.4%). An association supported by the three tree-making algorithms based on the 16S rRNA gene sequences indicating that YIM 131861 ^{T} clearly formed a

distinct lineage into the genus *Glaciibacter* (Fig. 1, Figures S1 and S2).

Genomic analyses

The draft genome of strain YIM 131861 ^{T} contained 30 contigs, with a total length of 3,402,707 bp and an N50 length of 299,953 bp (GenBank accession number SSSN00000000). The DNA G+C content of strain YIM 131861 ^{T}, determined from the genome, was 68.2 mol%. Strain YIM 131861 ^{T} genome was annotated with 3,313 genes, included 3,200 protein-coding genes, 3 rRNA genes, 47 tRNA genes, 3 ncRNA genes and 60 pseudogenes. In contrast, the draft genome of the reference strain *Glaciibacter superstes* NBRC 104264 ^{T} consists of 4,802,534 bp with an N50 contig length of 254,677 bp and a G+C content of 65.0 mol%. The ANI value between strain YIM 131861 ^{T} and *Glaciibacter superstes* NBRC 104264 ^{T} was 73.2% based on the draft genome sequence, which was lower than the 95.0% cut-off for species demarcation (Richter et al. 2009). The digital DNA–DNA hybridization value between strain YIM 131861 ^{T} and *Glaciibacter superstes* NBRC 104264 ^{T} was 19.9%, which was much lower than the threshold value (70%) recommended for distinguishing novel prokaryotic species.

Chemotaxonomic characterisation

The polar lipid profiles of strain YIM 131861 ^{T} were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and two unknown glycolipids (GL) (Figure S3), which was consistent with *Glaciibacter superstes* NBRC 104264 ^{T}, but one unknown glycolipid was absent in strain YIM 131861 ^{T}. The predominant menaquinones were MK-12 (45.0%) and MK-13 (55.0%), which was uniform with the genus *Glaciibacter*. The major cellular fatty acids (> 10%) were identified as *anteiso*-$C_{15:0}$ (37.6%), *iso*-$C_{16:0}$ (33.7%) and *anteiso*-$C_{17:0}$ (15.0%), the components of which were the same of the reference strain but the percentages were distinct. Furthermore, strain YIM 131861 ^{T} and the reference strain could also be distinguished by quantity and percentage of other minor fatty acids (Table 2). Strain YIM 131861 ^{T} contained

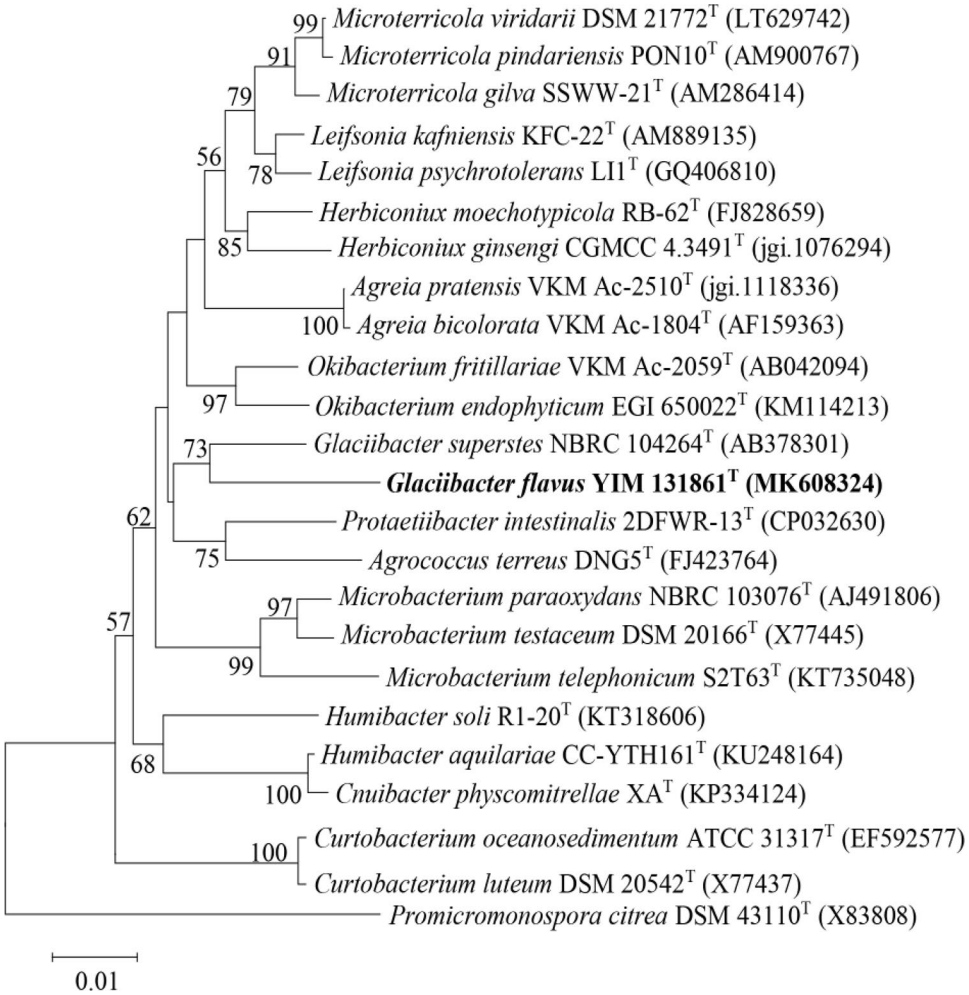

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain YIM 131861 ^{T} in relation to its nearest phylogenetical neighbours. Numbers at nodes indicate the level of bootstrap support (> 50%) based on 1000 resamplings. *Promicromonospora citrea* DSM 43110 ^{T} (X83808) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position

glutamic acid, glycine, alanine and 2,4-diaminobutyric acid as the peptidoglycan amino acids, and galactose, rhamnose, ribose and glucose as the whole-cell sugars. These results support that strain YIM 131861 ^{T} represents a novel species of the genus *Glaciibacter*.

Taxonomic conclusion

On the basis of the results of phenotypic, chemotaxonomic and phylogenetic analysis, strain YIM 131861 ^{T} exhibited typical characteristics of the genus *Glaciibacter*. However, genotypic, phenotypic, and chemotaxonomic analysis presented in the current study clearly show that the strain differs from the reference strain *Glaciibacter superstes* NBRC 104264 ^{T}. Taken together, strain YIM 131861 ^{T} represents a novel species of the genus *Glaciibacter*, for which the name *Glaciibacter flavus* sp. nov. is proposed.

Description of *Glaciibacter flavus* sp. nov.

Glaciibacter flavus (fla'vus. L. masc. adj. *flavus* yellow, referring to the colour of colonies of the organism).

Table 2 Cellular fatty acid profile of strain YIM 131861 ^{T} and closely related reference strain

Fatty acid	1	2
Saturated		
$C_{14:0}$	0.3	0.2
$C_{16:0}$	3.3	2.0
$C_{17:0}$	–	0.5
Branched saturated		
anteiso-$C_{15:0}$	**37.6**	**43.0**
anteiso-$C_{17:0}$	**15.0**	**37.7**
iso-$C_{15:0}$	4.3	2.4
iso-$C_{16:0}$	**33.7**	**10.0**
iso-$C_{17:0}$	1.2	1.0
Hydroxy		
$C_{15:0}$ 3-OH	1.3	–
Summed features*		
3	–	0.6
8	1.3	–

Strains: 1, YIM 131861 ^{T}; 2, *Glaciibacter superstes* NBRC 104264 ^{T}. All data were obtained from this study. Values are percentages of total fatty acids. The major fatty acids (greater than 10%) are shown bold. –, Not detected

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3, $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 8, $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$

Cells of strain YIM 131861 ^{T} are strictly aerobic, Gram-stain-positive, no-spore-forming, irregular short rod-shaped (0.4 – 0.5 × 1.0 – 1.6 μm in size) and motile with a polar flagellum. Strain YIM 131861 ^{T} is catalase positive and oxidase negative. Colonies are circular, smooth, moist, glistening and yellow on R2A medium. Growth occurs at 15 – 30 °C (optimum 25 °C), at pH 6.0 – 10.0 (optimum pH 7.0) and at 0 – 1.5% NaCl (optimum 1%). Urease activity, milk coagulation and peptonization, H_2S production, nitrate reduction, gelatine liquefaction and hydrolysis of cellulose and Tweens (20, 40, 60, 80) are negative, except for hydrolysis of starch. In the API 50CH test, acid is produced from glycerol, L-arabinose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, esculin citrate, D-cellobiose and glycogen, but not from D-ribose, D-xylose, D-maltose, xylitol and D-turanose. The cell-wall peptidoglycan contains 2,4-diaminobutyric acid as diamino acid and whole-cell sugars are galactose, rhamnose, ribose and glucose. The polar lipid profiles consist of diphosphatidylglycerol, phosphatidylglycerol and two unknown glycolipids. The predominant menaquinones are MK-12 and MK-13. Major fatty acids are *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$ and *anteiso*-$C_{17:0}$. The DNA G + C content of the type strain is 68.2 mol% based on the draft genome sequence.

The type strain, YIM 131861 ^{T} (= CGMCC 1.16588 ^{T} = NBRC 113572 ^{T}), was isolated from lichen collected from the South Bank Forest of the Baltic Sea. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence and the draft genome sequence are MK608324 and SSSN00000000 respectively.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02247-0.

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Authors' contributions DFA and LQJ performed the experiments and wrote the manuscript; JFI collected the lichen samples; KZ, GDL, XYW and LL analysed the data; MGJ identified the lichen samples; YJ guided the experiments and revised the manuscript; CLJ and LSW designed the study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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