Hyphomicrobium album sp. nov., isolated from mountain soil and emended description of genus Hyphomicrobium

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Abstract
A soil bacterium, designated XQ2T, was isolated from Lang Mountain in Hunan province, P. R. China. The strain is Gram stain negative, facultative anaerobic, and the cells are motile and rod-shaped. The 16S rRNA gene sequence of strain XQ2T shared the highest similarities with Hyphomicrobium sulfonivorans S1T (97.1%), Pedomicrobium manganicum ACM 3038T (95.9%) and Hyphomicrobium aestuarii DSM 1564T (95.4%) and grouped with H. sulfonivorans S1T. The average nucleotide identity (ANI) values and the DNA–DNA hybridization (dDDH) values between strain XQ2T and H. sulfonivorans S1T were 86.6% and 55.4% respectively. Strain XQ2T had a genome size of 3.91 Mb and the average G+C content was 65.1%. The major fatty acids (> 5%) were C18:1ω6c, C18:1ω7c, C19:0 cyclo ω8c, C16:0 and C18:0. The major respiratory quinone was Q-9 (82.8%) and the minor one was Q-8 (17.2%). The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, unidentified phospholipid and two unidentified lipids. On the basis of phenotypic, chemotaxonomic and phylogenetic characteristics, strain XQ2T represents a novel species of the genus Hyphomicrobium, for which the name Hyphomicrobium album sp. nov. is proposed. The type strain is XQ2T (= KCTC 82378T = CCTCC AB 2020178T). The genus description is also emended.

Keywords Hyphomicrobium · Hyphomicrobium album · Phylogenetic analysis · Polyphasic analysis

Introduction

The genus Hyphomicrobium was first proposed in 1899 using Hyphomicrobium vulgare NCIB 8968T as the first of a group of hyphal, budding bacteria (Stutzer and Hartleb 1899; Skerman et al. 1980). To date, a total of 11 Hyphomicrobium species have been studied and published (http://www.bacterio.net/hyphomicrobium.html). The cells of Hyphomicrobium strains are rod-shaped with pointed ends, or oval, egg-, or bean-shaped; produce monopolar or bipolar filamentous outgrowths (hyphae or prosthecae) of varying length and 0.2–0.3 μm in diameter (Gliesche et al. 2005). Compared to strains of a closely related genus Pedomicrobium, the strains of Hyphomicrobium are facultatively methylotrophic which are able to use some C1 compounds, such as methanol and methylamine hydrochloride as sole carbon sources, but they are not able to use a variety of organic acids and ethanol as sole carbon sources as Pedomicrobium strains do (Moore et al. 1984).

To investigate bacterial strains that may represent novel bacterial species, we isolated strains from soil of Lang Mountain, Shaoyang city, Hunan Province, P. R. China. Based on 16S RNA gene sequencing analysis results, a potential novel strain XQ2 was used for polyphasic analysis.
Materials and methods

Sample source and strain isolation

A soil sample was collected from the Pepper Peak of Lang Mountain (N 26° 20′ 26.44″ E 110° 45′ 37.48″), Shaoyang city, Hunan Province, P. R. China. The pH of the soil was 6.5. Strain XQ2 was isolated using the dilution-plating method after incubation on R2A agar at 28 °C for 10 days. Single bacterial colonies were picked and subcultivated several times to obtain a pure isolate.

Phylogenetic analysis

The nearly complete 16S rRNA gene fragment (1508 bp) of XQ2T was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCTCGTCTGCTAG-3′) and 1492R (5′-GGTTACCTTGTGACTCAG-3′) as described (Fan et al. 2008) and sequenced by the Tsingke Company (Beijing, PR China). This sequence is identical with the 16S rRNA gene sequence of the genome of strain XQ2T. The sequence was compared with the available sequences using the BLAST program (http://www.ncbi.nlm.nih.gov/Blast.cgi) and the close relatives were extracted in the EzTaxon-e server (Kim et al. 2012) and aligned using CLUSTAL X (Larkin et al. 2007). Multiple alignments and phylogenetic analysis were conducted using MEGA version 6.0 (Tamura et al. 2013). Phylogenetic trees were reconstructed on the basis of maximum-likelihood (ML) (Felsenstein 1981), neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-parsimony (MP) (Nei and Kumar 2013) methods in which Methylobacterium organophilum was used as an outgroup.

To further explore the genomic relationships among strain XQ2T and the related members in genus Hyphomicrobium, the up-to-date bacterial core gene set (UBCG) and pipeline programs were utilized for phylogenetic tree reconstruction as described by Na et al. (2018).

Genome sequencing and analysis

Since strain XQ2T exhibited the highest 16S rRNA gene similarity to Hyphomicrobium sulfonivorans S1T (97.1%), the DNA of the two strains were randomly fragmented by ultrasonic crusher for sequencing. The library preparation was performed using the Illumina TruSeq DNA Sample Prepare kit. Pair-end sequencing was performed on an Illumina HiseqX system by Wuhan Frasergen Bioinformatics Co., Ltd (Hubei, P. R. China). Sequenced reads were assembled using SPAdes v3.11.1 (http://cab.spbu.ru/software/spades/). Gene prediction was carried out using Prodigal-2.6.2 (https://github.com/hyatt/Prodigal/wiki). The draft genomes of strain XQ2T and H. sulfonivorans S1T were submitted to NCBI and annotated using Prokaryotic Genome Annotation Pipeline.

To detect the relationship among strain XQ2T and H. sulfonivorans S1T, average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) were performed. The ANI values between strain XQ2T and H. sulfonivorans S1T were analyzed using the web version of the ANI calculator (www.ezbiocloud.net/tools/ani) (Yoon et al. 2017), whereas digital DNA–DNA hybridization (dDDH) was analyzed by a webserver (http://ggdc.dsmz.de/ggdc.php) (Meier-Kolthoff et al. 2013). Cluster of Orthologous Groups of proteins (COG) (http://weizhong-lab.ucsd.edu/webMGA/server/cog/) was used to analyze the protein functional categories with an E-value is 10–10 (Tatusov et al. 2000).

Morphological and physiological analysis

To conduct morphology, physiology and biochemistry analysis, strain XQ2T and the related strains, H. sulfonivorans S1T and H. aestuarii DSM 1564T, were cultured in R2A broth or on R2A agar at 28 °C, unless otherwise indicated. The Gram reaction was determined using a Gram staining kit (BASO Taiwan) and the KOH (3%) lysis methods (Vila et al. 1992). Cellular morphology was observed using a scanning electron microscope (JSM-6390, JEOL, Japan) and flagella were examined by transmission electron microscopy (H-7650; Hitachi) using cells cultivated on R2A (Difco) at 28 °C for 14 days. Endospore formation was observed using a phase-contrast microscope (BX51M; Olympus) when cells were cultivated on R2A plates at 28 °C for 7 days. Anaerobic growth was investigated using an anaerobic chamber (Mitsubishi Gas Chemical) on R2A plates at 28 °C for 2 weeks. Motility tests were observed using R2A with 0.3% (w/v) agar at 28 °C. Growth at different temperatures (4, 15, 20, 29, 37, 42, 45, 47, 49 and 50 °C), salt tolerance with 0–5% (w/v) NaCl (1% intervals) and growth at different pH values (4–10) at 1pH unit interval were measured in R2A broth at 28 °C for 7 days. The pH was adjusted using the following buffer systems: pH 4.0–7.0, 0.1 M citric acid/0.2 M Na2HPO4; pH 8.0–9.0, 0.2 M Tris/0.2 M HCl; pH 10.0, 0.05 M NaHCO3 /0.1 M NaOH (Vila et al. 1992). Growth was also tested in TSB (tryptic soy broth), NB (nutrient broth) and LB (Luria–Bertani) media.

Oxidase activity was assessed by oxidation of tetramethyl-p-phenylenediamine, and catalase activity was determined by production of bubbles after adding 3% H2O2 (Cowan and Steel 1965). Production of phenylalanine deaminase was tested using 10% (w/v) FeCl3 solution as described by Vila et al. (1992). Hydrolyses of starch, gelatin, casein, DNA, CM-cellulose, Tweenes 20, 40, 60 and 80 were determined as described by Cowan and Steel (1965). Production of H2S and indole; Voges–Proskauer and Methyl Red reactions were tested. Dong and Cai (2001). Acid production from various
carbohydrates was determined according to the protocols of Leifson (1963). Utilization of sole carbon sources was tested using ID 32GN systems. Enzyme activities were tested using API ZYM systems and additional physiological and biochemical characteristics were examined using API 20NE systems according to the manufacturer’s instructions (Bio-Mérieux, France). Some of these tests were confirmed in combination with traditional methods (Dong and Cai 2001).

Chemotaxonomic analysis

The fatty acids of strains XQ2<sup>T</sup> and the *H. sulfonivorans* S1<sup>T</sup> and *H. aestuarii* DSM 1564<sup>T</sup> were analyzed by gas chromatography according to the protocol of MIDI (version 6.1 and TSBA library version 6.1) (Sasser 1990). These strains were cultivated on R2A plates at 28 °C and collected when the bacteria reached their mid-exponential phase. The polar lipids of strain XQ2<sup>T</sup>, *H. sulfonivorans* S1<sup>T</sup> and *H. aestuarii* DSM 1564<sup>T</sup> were analyzed in this study by two-dimensional thin-layer chromatography (TLC) (Minnikin et al. 1984). The respiratory quinones of strain XQ2<sup>T</sup> were extracted from lyophilized cells (Collins et al. 1977) grown in R2A medium at 28 °C for 2 days, purified by TLC and then analyzed by HPLC (Xie and Yokota 2003).

Results and discussion

Phylogeny analysis

Strain XQ2<sup>T</sup> showed the highest similarities to *H. sulfonivorans* S1<sup>T</sup> (97.1%), *P. manganicum* ACM 3038<sup>T</sup> (95.9%), and *H. aestuarii* DSM 1564<sup>T</sup> (95.4%). The NJ tree clustered strain XQ2<sup>T</sup> with *H. sulfonivorans* S1<sup>T</sup> (Fig. 1). The ML and MP trees showed similar results (Figs. S1 and S2, available in the online version). Therefore, the two most closely related species, *H. sulfonivorans* S1<sup>T</sup>, and the *H. aestuarii* DSM 1564<sup>T</sup> were obtained and analyzed together in this study. The core-genomic phylogenetic tree based on the amino acid sequences of 88 protein clusters showed that strain XQ2<sup>T</sup> formed a distinct phylogenetic position in genus *Hyphomicrobium* (Fig. 2).

Genome characterization

The genome size of XQ2<sup>T</sup> was 3.91 Mb, with 332 × depth of coverage and a DNA G+C content of 65.1%, a total of 3 contigs (> 1000 bp) contained 3,659 protein-coding genes. After comparing the genome sequence with those in ResFinder,
PlasmidFinder and PointFinder databases, no plasmid was found in the genome of strain XQ2T. The genome of strain *H. sulfonivorans* S1T was 3.55 Mb including 12 contigs with N50 of 1,057,257, 6 rRNA operons, 3144 coding sequences and 404× depth of coverage. These qualities meet the standards of genome data for the taxonomy of prokaryotes proposed in 2018 (Chun et al. 2018). The genomic information of strains XQ2T (WMBQ01000000) and *H. sulfonivorans* S1T (PZPO00000000) are listed in Table S1. Based on the genome sequences comparison, an orthoANI value of 86.6% between strain XQ2T and *H. sulfonivorans* S1T was obtained, which was significantly less than the threshold 95–96% ANI value for species delineation (Goris et al. 2007). In addition, the estimated genome-sequence-based digital DNA–DNA hybridization (dDDH) values were calculated (Meier-Kolthoff et al. 2013). The dDDH values between strain XQ2T and *H. sulfonivorans* S1T was 55.4%, respectively, which were below the threshold of 70% for species delineation (Wayne et al 1987). The distribution of proteins into COGs functional categories are shown in Table S2.

Morphological and physiological characteristics

The different characteristics of strain XQ2T with the closely related strains are shown in Table 1. In addition, compared to *P. manganicum* ACM 3038T, strain XQ2T and other two strains of *Hyphomicrobium* were able to use methanol and methylamine hydrochloride as sole carbon sources, but were not able to use variety of organic acids (such as capric acid, succinic acid and citric acid) and ethanol as sole sources as *P. manganicum* ACM 3038T did (Moore et al 1984; Gebers 1981).

Chemotaxonomic characteristics

The major fatty acids (> 5%) of strain XQ2T were summed featured 8 (C18:1ω6c and/or C18:1ω7c, 73.3%), which were similar to other species of the genus *Hyphomicrobium* (Table 2). The respiratory quinones of strain XQ2T were Q-9 (82.8%) and Q-8 (17.2%) (Fig. S4), which were a characteristic feature of *Hyphomicrobium* members. The polar lipids of strain XQ2T were diphasphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine which were similar to that in *H. sulfonivorans* S1T and *H. aestuarii* DSM 1564T (Fig. S5).

In conclusion, the results of physiological and biochemical experiments showed that the new strain XQ2T had many common traits with the related strains such as assimilating N-acetyl-glucosamine and using methanol and methylamine as sole carbon sources. Chemotaxonomically, the results of respiratory quinones, polar lipids and fatty acids were also consistent with the characteristics of *Hyphomicrobium*. But strain XQ2T could be differentiated from the relatives by phylogenetic distance and the ability to assimilate *L*-arabinose, *D*-mannose and *D*-maltose. According to phylogenetic, physiological and chemotaxonomic data, as well as the results of ANI and dDDH, we consider that strain XQ2T representing a novel species of the genus *Hyphomicrobium*, for which the name *Hyphomicrobium album* sp. nov. is proposed.

**Description of *Hyphomicrobium album* sp. nov.**

*Hyphomicrobium album* (al’bum. L. neut. adj. album, white, referring to the color of the colonies).

Gram stain negative and facultative anaerobic. Cells are rod shaped with bud forming at the tip of a prosthecate. Grow heterotrophically and aerobically on melibiose, arabinose, valerate, citrate, histidine, gluconate, butyrate, benzoate, sucrose, maltose, acetate, lactate, alanine, gluconate, glycogen, and yeast extract. The major respiratory quinone is Q-9 and the minor one is Q-8. The major fatty acid is summed feature 8 (C18:1ω7c and/or C18:1ω6c). The polar lipids are diphasphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, unidentified phospholipid and unidentified lipids. The G+C content of its genomic DNA is 65.1 mol%.
The type strain is XQ2T (= KCTC 82378T = CCTCC AB 2020178T), isolated from Lang mountain, Shaoyang city, Hunan Province, PR China.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the draft genome of Hyphomicrobium album XQ2T are MN647626 and NZ_WMBQ01000000, respectively.

**Table 1** Different phenotypic characteristics among strain XQ2T and the related type strains

| Characteristic                        | 1   | 2        | 3   |
|--------------------------------------|-----|----------|-----|
| Colony pigmentation                  | White | Brown | Yellow |
| Temperature range for growth (°C)    | 16–30 | 15–37 | 5–45 |
| NaCl range for growth (% w/v)       | 0–0.5 | 0–1.5 | 0–5.5 |
| Catalase                             | +    | +       | –   |
| Nitrate reduction                    | +    | –       | +   |
| L-Tryptophane                        | –    | +       | +   |
| Starch                               | –    | +       | +   |
| Tween 80                             | +    | –       | –   |
| Cystine arylamidase                  | –    | +       | +   |
| Tryptsin                             | –    | +       | +   |
| β-Glucosidase                        | +    | +       | –   |
| N-Acetyl-β-glucosaminidase           | +    | +       | –   |
| α-Chymotrypsin                       | +    | +       | –   |
| Urease                               | +    | +       | –   |
| 4-Nitrophenyl-β-d-galactopyranoside  | +    | –       | +   |
| Methanol                             | +    | +       | –   |
| Methylamine hydrochloride            | +    | +       | –   |
| Ethanol                              | –    | –       | –   |
| Capric acid                          | –    | –       | –   |
| Succinic acid                        | –    | –       | –   |
| Citric acid                          | –    | –       | –   |
| d-Glucose                            | –    | –       | –   |
| d-Ribose                             | –    | –       | –   |
| L-Arabinose                          | –    | –       | –   |
| d-Mannose                            | +    | –       | –   |
| d-Mannitol                           | +    | +       | –   |
| d-Maltose                            | +    | +       | +   |
| G+C mol%                             | 65.1 | 62.0    | 64.0 |

1, Strain XQ2T; 2, *H. sulfonivorans* S1T; 3, *H. aestuarii* DSM 1564T. The results were from this study unless otherwise mentioned. +, positive; –, negative; ND, not determined/no data available. All strains are positive for: the production of H2S, the activity of catalase, esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, b-galactosidase and a-glucosidase, the assimilation of d-glucose, d-ribose and d-(+)-galactose. All strains are negative for: the production of indole, the hydrolysis of Tween 80, DNA, CM-cellulose, aesculin ferric citrate, l-arginine and urea, the activity of oxidase and phenylalanine deaminase and acid production from rhamnose, l-(−)-sorbitol and inositol.

**Table 2** Fatty acids composition of strain XQ2T and the phylogenetically related strains

| Fatty acid | 1   | 2    | 3    |
|------------|-----|------|------|
| Saturated  | C<sub>16:0</sub> | 8.3  | 2.6  | 4.3  |
|            | C<sub>18:0</sub> | 6.6  | 2.0  | 2.4  |
| Unsaturated| C<sub>19:0</sub> cyclo o8c | 6.9  | 16.1 | 7.7  |
|            | C<sub>18:0</sub> iso | –   | –   | 2.9  |
| Hydroxy    | C<sub>14:0</sub> 3-OH | –   | –   | –    |
|            | C<sub>16:0</sub> 3-OH | 1.9  | 2.5  | 1.3  |
|            | C<sub>18:0</sub> 3-OH | 1.1  | 1.4  | –    |
| Summed feature 8* | 73.3 | 73.2 | 76.9 |
| Summed feature 2a | –   | –   | 3.0  |

1, Strain XQ2T; 2, *H. sulfonivorans* S1T; 3, *H. aestuarii* DSM 1564T. All of the results were from this study. –, <1%

Summed feature 8 comprises C<sub>18:1ω6c</sub> and/or C<sub>18:1ω7c</sub>

Summed feature 2 comprises C<sub>14:0</sub> 3-OH and/or C<sub>16:1 iso I</sub>

*a Summed features represent groups of two fatty acids which could not be separated by GLC and the MIDI system.

**Emended description of the genus Hyphomicrobium Stutzer and Hartleb 1899 (Approved Lists 1980)**

The description is as given previously (Urakami and Komagata 1987) with the following addition. Fatty acids of *Hyphomicrobium* strains contain a large amount of straight-chain unsaturated C<sub>18:1</sub> acid, and some of the strains also have 3-OH C<sub>14:0</sub> and 3-OH C<sub>16:0</sub> hydroxy acids. All strains of *Hyphomicrobium* have ubiquinone Q-9, some of the strains also have Q-8 or Q-10. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylethanolamine.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02473-6.

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**Declarations**

**Conflict of interest** The authors declare that there are no conflicts of interest.
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