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

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Abstract

A soil bacterium, designated XQ2^T, was isolated from Lang Mountain in Hunan province, P. R. China. The strain is Gram-stain-negative, facultative anaerobic, and the cells are motile, rod-shaped. The 16S rRNA gene sequence of strain XQ2^T shared the highest similarities with *Hyphomicrobium sulfonivorans* S1^T (97.1%), *Pedomicrobium manganicum* ACM 3038^T (95.9%) and *Hyphomicrobium aestuarii* DSM 1564^T (95.4%) and grouped with *H. sulfonivorans* S1^T. The average nucleotide identity (ANI) values and the DNA–DNA hybridization (dDDH) values between strain XQ2^T and *H. sulfonivorans* S1^T were 86.6% and 55.4% respectively. Strain XQ2^T had a genome size of 3.91 Mb and the average G+C content was 65.1%. The major fatty acids (> 5%) were $C_{18:1}\omega6c$, $C_{18:1}\omega7c$, $C_{19:0}$ cyclo $\omega8c$, $C_{16:0}$ and $C_{18:0}$. 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 XQ2^T represents a novel species of the genus *Hyphomicrobium,* for which the name *Hyphomicrobium album* sp. nov. is proposed. The type strain is XQ2^T (= KCTC 82378^T = CCTCC AB 2020178^T). The emended description of genus *Hyphomicrobium* is also described.

Introduction

The genus *Hyphomicrobium* was first proposed in 1898 using *Hyphomicrobium vulgare* ATCC 27500^T as the first of a group of hyphal, budding bacteria (Steigerwalt et al. 1976). To date, a total of 13 *Hyphomicrobium* species have been published (http://www.bacterio.net/hyphomicrobium.html). The cells of *Hyphomicrobium* strains are rod shaped with flagella, and cells are reproduced by budding at the tip of cell prosthecae (Christine et al. 2013, Elena et al. 2002, Izumi et al. 1982, Johnson and Weisrock 1969, McDonald et al. 2001, Moore et al. 1984, Steigerwalt et al. 1976, Urkami et al. 1995, Urakami and Komagata 1987, Urakami and Komagata 1986, Urakami et al. 1985, Urakami and Komagata 1979). The major fatty acids summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$). Comparing to strains of a closely 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 variety of organic acids and ethanol as sole carbon sources as *Pedomicrobium* strains do (Moore et al. 1984). The major ubiquinone of *Hyphomicrobium* strains is Q-9 and the minor one is Q-8 (Christine et al. 2013, Elena et al. 2002, Izumi et al. 1982, Johnson and Weisrock 1969, McDonald et al. 2001, Moore et al. 1984, Steigerwalt et al. 1976, Urkami et al. 1995, Urakami and Komagata 1987, Urakami and Komagata 1986, Urakami et al. 1985, Urakami and Komagata 1979). The polar lipids of *Hyphomicrobium* haven't been tested so far.

Here, a novel *Hyphomicrobium* strain XQ2^T was isolated and used for polyphasic analyses. Although the strain belongs to the genus *Hyphomicrobium*, it differs from the other types in several genetic, phenotypic, and chemotaxonomic traits. Therefore, it is proposed that a new species name of *Hyphomicrobium album* sp. nov. should be established.

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 (in the water) of the soil was 6.5. Strain XQ2^T was isolated using the dilution-plating method after incubation on R2A agar at 28 °C for 10 days. Colonies were picked and single colonies were activated several times using the same media to obtain a pure isolate.

Phylogenetic analysis

The nearly complete 16S rRNA gene fragment (1,508 bp) of XQ2^T was amplified using the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-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 XQ2^T. 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 1981) and maximum-parsimony (MP) (Nei and Kumar 2013) methods and *Enterobacter huaxiensis* was used as an out-group.

In order to further explore the genomic relationships among strain XQ2^T 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. (Na et al. 2018).

Genome sequencing and analysis

For genomic analysis, since strain XQ2^T exhibited the highest 16S rRNA gene similarity to *H. sulfonivorans* S1^T (97.1%), the DNA of two strains were randomly fragmented by ultrasonic crusher. 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/hyattpd/prodigal/wiki). The draft genomes of strain XQ2^T and *H. sulfonivorans* S1^T were submitted to NCBI and annotated using Prokaryotic Genome Annotation Pipeline.

To detect the relationship among strain XQ2^T and *H. sulfonivorans* S1^T, average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) were performed. The ANI values between strain XQ2^T and *H. sulfonivorans* S1^T were analyzed using the web version of the ANI calculator (www. ezbiocloud. net/tools/ani) (Yoon et al. 2017b) 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 XQ2^T and other related strains *H. sulfonivorans* S1^T and *H. aestuarii* DSM 1564^T were cultured in R2A broth or on R2A agar at 28 ºC, unless otherwise indicated. The Gram reaction was determined using a Gram staining kit (BASO) (Tarrand and Gröschel 1982) 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 ℃ for 14 days. Endospore formation was observed using a phase-contrast microscope (BX51M; Olympus) when cells were cultivated on R2A plates at 28 ℃ for 7days. Anaerobic growth was investigated using an anaerobic chamber (Mit-subishi Gas Chemical) on R2A plates at 28 ℃ for 2 weeks. Motility tests were observed using R2A with 0.3% (w/v) agar at 28 ℃. Growth at different temperatures (4, 15, 20, 29, 37, 42, 45, 47, 49 and 50 ℃), 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 ℃ 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 Na_2HPO_4; pH 8.0–9.0, 0.2 M Tris/0.2 M HCl; pH 10.0, 0.05 M $NaHCO_3$ /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% H_2O_2 (Cowan and Steel 1965). Production of phenylalanine deaminase was tested using 10% (w/v) $FeCl_3$ solution as described by Vila et al. (Vila et al. 1992). Hydrolyses of starch, gelatin, casein, DNA, CM-cellulose, Tweens 20, 40, 60 and 80 were determined as described by Cowan and Steel (Cowan and Steel 1965). Production of H_2S and indole, Voges–Proskauer and Methyl Red reactions were tested according to the methods of this article (Dong and Cai 2001). Acid production from various carbohydrates was determined according to the protocols of Leifson (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 (BioMérieux, France). Some of these tests were confirmed in combination with traditional methods (Sasser 1990).

Chemotaxonomic analysis

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

Results And Discussion

Phylogeny analysis

Strain XQ2^T showed the highest similarities to *H. sulfonivorans* S1^T (97.1%), *P. manganicum* ACM 3038^T (95.9%), *H. aestuarii* DSM 1564^T (95.4%). The NJ tree clustered strain XQ2^T with *H. sulfonivorans* S1^T (Fig. 1). The ML and MP trees showed similar results (Fig. S1 and S2, available in the online version). Therefore, the two most closely related species, *H. sulfonivorans* S1^T, and the *H. aestuarii* DSM 1564^T were obtained and analyzed together in this study (*P. manganicum* ACM 3038T wasn't available to purchase). The core-genomic phylogenetic tree based on the amino acid sequences of 88 protein clusters showed that strain XQ2^T formed a distinct phylogenetic position in genus *Hyphomicrobium* (Fig. 2).

Genome characterization

The genome size of XQ2^T was 3.91 Mb, with 332x depth of coverage and a DNA G+C content of 65.1 %, a total of 3 contigs (>1,000 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 XQ2^T. The genome of strain *H. sulfonivorans* S1^T was 3.55 Mb including 12 contigs with N50 of 1,057,257, 6 rRNA operons, 3,144 coding sequences and 404x 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 XQ2^T (WMBQ01000000) and *H. sulfonivorans* S1^T (PZPO00000000) are listed in Table S1. Based on the genome sequences comparison, an orthoANI value of 86.6% between strain XQ2^T and *H. sulfonivorans* S1^T 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 as described by Meier-Kolthoff et al. (Meier et al. 2013). The dDDH values between strain XQ2^T and *H. sulfonivorans* S1^T was 55.4%, respectively, which were below the threshold of 70% for species delineation (Na et al. 2018). The distribution of proteins into COGs functional categories are shown in Table S2.

Morphological and physiological characteristics

The differences in phenotypic characteristics between XQ2^T and four reference species are given in Table 1. The cells of strain XQ2^T were rod-shaped (0.4-0.6 μm in width and 1.0-1.2 μm in length) (Fig. S3), motile, Gram-stain-negative and facultative anaerobic, oxidase and catalase positive. Colonies grown on R2A agar plates for 14 days were round, rough, convex, milky white and less than 2.0 mm in diameter. The strain could grow on R2A agar, TSB, but did not grow on LB agar, 1/10 TSA agar, and NB agar. The strain grows heterotrophically and aerobically on melibiose, arabinose, valerate, citrate, histidine, gluconate, butyrate, benzoate, sucrose, maltose, acetate, lactate, alanine, gluconate, glycogen, and yeast extract. The strain could grow at 15-37 ℃ and pH 5.5-9.5, and the optimum growth temperature and pH were 25 °C and 7, respectively. The tolerance of NaCl was 1%. It could hydrolyze Tween 80 and gelatin, but not Tween 20, Tween 60, starch, and casein. The different characteristics of strain XQ2^T with the closely related strains are shown in Table 1. Compared with the closest related strain *H. sulfonivorans* S1^T, strain XQ2^T had the ability of assimilation of 4-nitrophenyl-*β*-D-galactopyranoside, D-glucose, L-arabinose, D-mannose, D-maltose. The ability to assimilate L-arabinose, D-mannose and D-maltose were positive for strain XQ2^T and negative for the other closely related strains. In addition, comparing to *P. manganicum* ACM 3038^T, strain XQ2^T 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 3038^T did (Moore et al 1984, Mikrobiologie et al. 1981).

Chemotaxonomic characteristics

The major fatty acids (> 5%) of strain XQ2^T were summed feature 8 (C18:1 *ω*6*c* and/or C18:1 *ω*7*c*, 73.3%), which were generally similar to the results reported for other species of the genus *Hyphomicrobium* (Table 2). The respiratory quinones of strain XQ2^T were Q-9 (82.8%) and Q-8 (17.2%) (Fig. S4), which were a characteristic feature of *Hyphomicrobium* members. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, unidentified phospholipid and unidentified lipids., which were similar to most of the *Hyphomicrobium* strains (Fig. S5).

In conclusion, the results of physiological and biochemical experiments showed that the new strain XQ2^T 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 XQ2^T 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 XQ2^T 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. Growth was observed at 15–37 ℃ (optimum at 25 ℃), pH 5.5–9.5 (optimum at pH 7.0–8.0) and with 0–1% (w/v) NaCl (optimum without NaCl). 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 was Q-8. The major fatty acids are summed feature 8 ($C_{18:1}$ *ω*7*c* and/or $C_{18:1}$ *ω*6*c*). The predominant polar lipids are diphosphatidylglycerol; phosphatidylglycerol; phosphatidylethanolamine; phosphatidylcholine; unidentified phospholipid and unidentified lipids.

The type strain is XQ2^T (= KCTC 82378^T = CCTCC AB 2020178^T), isolated from Lang mountain, Shaoyang city, Hunan Province, PR China. The G+C content of its genomic DNA is 65.1 mol %. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the draft genome of *Hyphomicrobium album* XQ2^T are MN647626 and NZ_WMBQ01000000, respectively.

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. Major fatty acids are summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Major respiratory quinone was Q-9 and the minor one was Q-8. The major polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, unidentified phospholipid. The type species is *Hyphomicrobium vulgare* ATCC 27500^T.

Declarations

Footnote: The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain XQ2^T is MN647626. The genome numbers of strain XQ2^T is NZ_WMBQ01000000, respectively.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Tables

Table 1 Different phenotypic characteristics among strain XQ2^T and the related type strains.

1, Strain XQ2^T; 2, *H. sulfonivorans* S1^T; 3, *H. aestuarii* DSM 1564^T; 4, *H. vulgare* ATCC 27500^T (type species strain); 5, *P. manganicum* ACM 3038^T. The results were from this study unless otherwise mentioned. +, positive; -, negative; ND, not determined/no data available. Data of isolation source, growth temperature, pH and NaCl range and DNA G+C contents of the four reference strains were taken from previous studies [16,17,18]. All strains are positive for: the production of H_2S, 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-(+)-gatactose. 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-(-)-sorbose and inositol.

Characteristic	1	2	3	4*	5§
Colony pigmentation	white	brown	yellow	brown	brown-black
Temperature range for growth (°C)	16-30	15-37	5-45	15-37	21-36
NaCl range for growth (%, w/v)	0-0.5	0-1.5	0-5.5	0-5.5	0-0.5
Catalase	+	+	-	+	+
Nitrate reduction	+	-	+	+	-
L-tryptophane	-	+	+	ND	ND
Starch	-	+	+	ND	ND
Tween 80	+	-	-	ND	ND
Cystine arylamidase	-	+	+	-	ND
Trypsin	-	+	+	+	ND
β-glucosidase	+	-	-	+	ND
N-acetyl-β-glucosaminidase	+	+	-	+	ND
α-chymotrypsin	+	+	-	-	ND
Urease	+	+	+	-	
4-nitrophenyl-*β*-D-galactopyranoside	+	-	-	ND	ND
methanol	+	+	+	+	-
methylamine hydrochloride	+	+	+	ND	-
ethanol	-	-	-	-	+
capric acid	-	-	-	ND	+
succinic acid	-	-	-	ND	+
citric acid	-	-	-	ND	+
D-glucose	-	-	-	ND	+
D-ribose	-	-	-	-	+
L-arabinose	+	-	-	-	-
D-mannose	+	-	-	-	-
D-mannitol	+	-	-	-	-
D-maltose	+	-	+	-	-
G+C mol%	65.1	62.0	64.0	61.1	66.0

* The data are taken from Li et al. [35].

§All data are taken from Moore et al and Sly et al. respectively [7,37].

Table 2. Fatty acids composition of strain XQ2^T and the phylogenetically related strains. 1, Strain XQ2^T; 2, *H. sulfonivorans* S1^T; 3, *H. aestuarii* DSM 1564^T; 4, *H. vulgare* ATCC 27500^T. All of the results were from this study unless otherwise mentioned. -, < 1%.

Fatty acid	1	2	3	4§
Saturated:				
$C_{16:0}$	8.3	2.6	4.3	2.5
$C_{18:0}$	6.6	2.0	2.4	1.5
Unsaturated:				
$C_{19:0}$ cyclo $\omega 8c$	6.9	16.1	7.7	-
$C_{18:0}$ iso	-	-	2.9	-
Hydroxy:				
$C_{14:0}$ 3-OH	-	-	-	5.6
$C_{16:0}$ 3-OH	1.9	2.5	1.3	2.5
$C_{18:0}$ 3-OH	1.1	1.4	-	-
Summed feature 8*	73.3	73.2	76.9	87.9
Summed feature 2*	-	-	3.0	5.6

§All data are taken from Teizi et al. [8].

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

Summed feature 8 comprises $C_{18:1}\omega 6c$ and/or $C_{18:1}\omega 7c$;

Summed feature 2 comprises $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I;

Figures

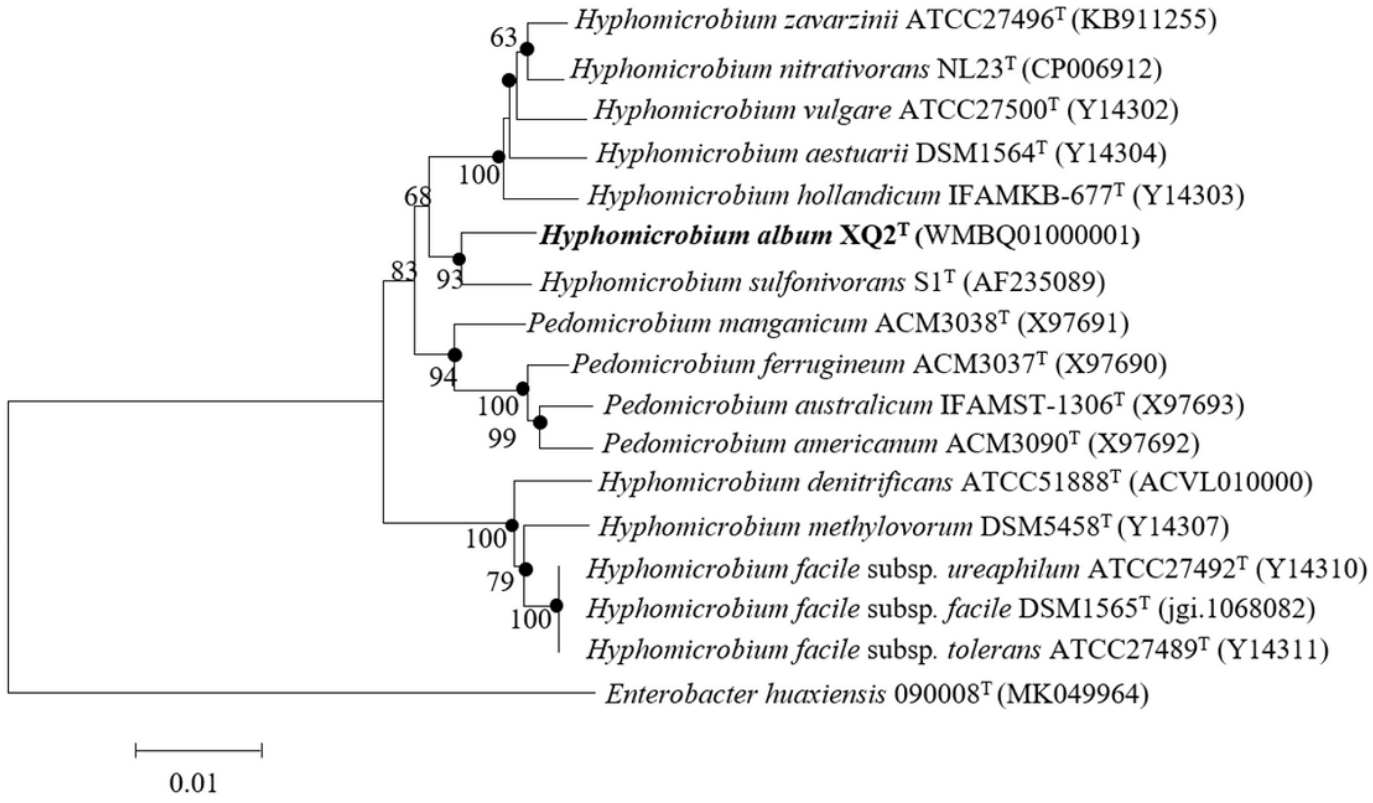

Figure 1

A neighbor-joining tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain XQ2T and the closely related strain of the genus Hyphomicrobium. Bootstrap values (> 50%) based on 1,000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position. Filled circles indicate that the corresponding nodes were also recovered in trees reconstructed by the maximum-parsimony and maximum-likelihood methods. Enterobacter huaxiensis 090008T (MK049964) was used as an outgroup.

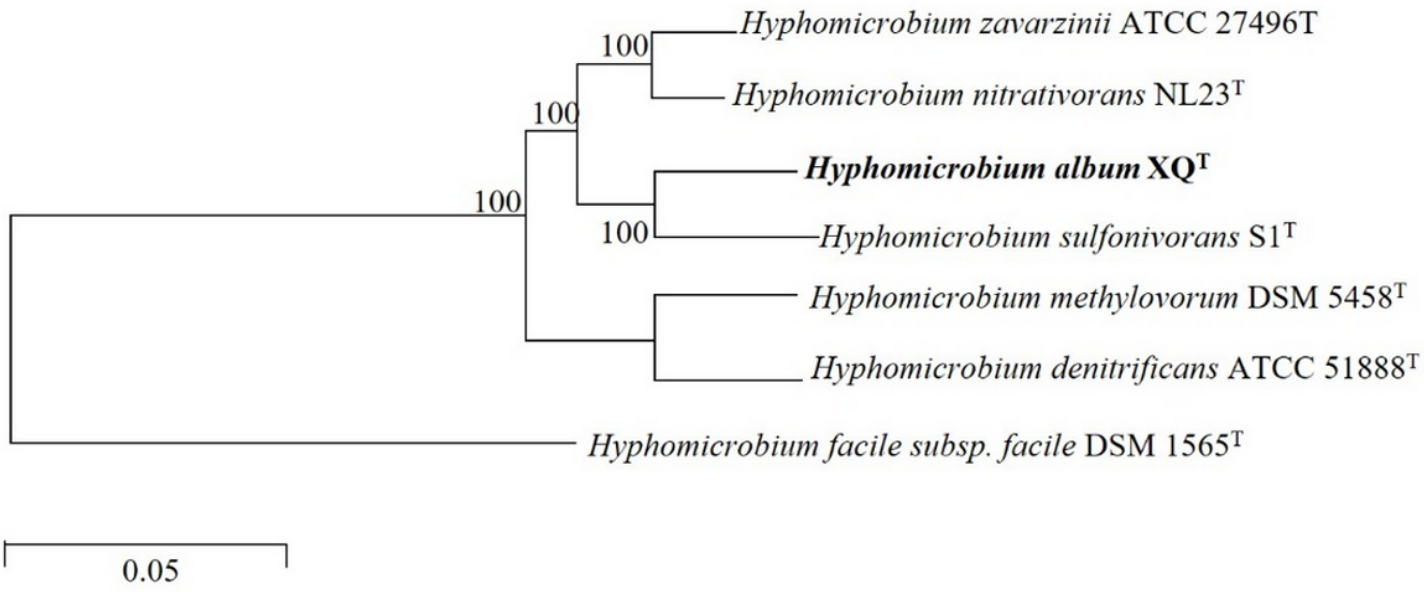

Figure 2

A phylogenetic tree inferred using UBCGs (concatenated alignment of 88 core genes) showing the relationship of Hyphomicrobium album XQ2T and the closely related taxa within the genus Hyphomicrobium. The number of single gene trees supporting a branch in a UBCG tree is calculated and designated the gene support index (GSI). The GSIs (> 50) are given at branching points. Bar, 0.05 substitutions per position.

Supplementary Files

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- 3Supplements.pdf