Neobacillus paridis sp. nov., an endophyte of Paris polyphylla Smith var. yunnanensis

Peng-Chao Zhan · Cong-Jian Li · Zhen Zhang · Rui-Feng Mao · Jing-Ran Liu · Xing-Wang Jiang · Xiao-Yang Zhi · Ling-Ling Yang

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Abstract
A novel endophytic strain, designated YIM B02564T, was isolated from the root of Paris polyphylla Smith var. yunnanensis obtained from Yunnan Province, southwest China. By using a polyphasic approach, cells of the strain were characterized as facultative anaerobic, Gram-positive and rod-shaped. The growth conditions of the strain were found to occur at 20–55 °C (optimum, 30 °C), pH 6.0–9.0 (optimum, pH 7.0). Strain YIM B02564T can tolerate 2% NaCl concentration. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM B02564T belonged to the genus Neobacillus and the 16S rRNA gene sequence similarity values of strain YIM B02564T to the type strains of members of this genus ranged from 95.6 to 97.8%. The DNA G+C content of strain YIM B02564T calculated from the whole genome sequence was 41.6 mol%. Values of the ANI and the dDDH between strain YIM B02564T and its closely related Neobacillus species were below 77.9% and 21.5%. Strain YIM B02564T contained MK-7 as the major menaquinone, iso-C15:0 and anteiso-C15:0 as the major fatty acids. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminophospholipid and four unidentified lipids. It contained meso-diaminopimelic acid in the cell-wall peptidoglycan. On the basis of polyphasic analysis, strain YIM B02564T could be differentiated genotypically and phenotypically from recognized species of the genus Neobacillus. The isolate therefore represents a novel species, for which the name Neobacillus paridis is proposed. The type strain is YIM B02564T (= JCM 34668T = CGMCC 1.18655T).

Keywords Neobacillus paridis sp. nov. · Paris polyphylla Smith var. yunnanensis · Polyphasic taxonomy

Abbreviations
R2A Reasoner’s 2A
NA Nutrition agar
TSA Tryptic soy agar
NJ Neighbor-joining
ML Maximum-likelihood
GCM The Global Catalogue of Microorganisms
CSI Conserved signature indel
MK Menaquinone
AAI Average amino acid identity
ANI Average nucleotide identity
dDDH Digital DNA–DNA hybridization

Introduction
The genus Neobacillus and other five genera were proposed by dividing the former genus Bacillus based on phyllogenomic and comparative genomic approaches (Patel and Gupta 2020). At the time of writing, this genus harbors 16 valid published species isolated from diverse environmental habitats, including soil, human gut and plant roots (https://lpsn.dsmz.de/genus/neobacillus). During an investigation of endophytic bacterial communities associated with traditional Chinese medicinal plants in Yunnan Province, southwest China, strain YIM B02564T was isolated from the surface-sterilized root of Paris polyphylla Smith var. yunnanensis. Its harvested rhizomes have become the indispensable component of more than 70 popular patented medicines for treatment of carbuncle, sore throat, venomous snake bites, and
traumatic pain in China (Qin et al. 2018). In this work, based on a polyphasic approach, we describe the characterizations of a novel strain YIM B02564T isolated from this medicinal plant and report that this strain represents a novel species of the genus Neobacillus.

Materials and methods

Bacterial isolation and maintenances

Healthy roots of Paris polyphylla var. yunnanensis were collected from Shilin County, Yunnan Province, southwest China for further sterilizing and pulverizing before distribution on R2A medium which was described by Yang et al. (2016). During 10 days’ incubation at 25 ºC, the colonies obtained were re-streaked on the same medium until pure colonies were obtained and stored both on R2A slants at 4 ºC and in 15% (v/v) glycerol at – 80 ºC for long-term preservation.

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA of strain YIM B02564T was extracted using a genomic DNA extraction kit (Tiangen, China) whose 16S rRNA gene sequence of strain YIM B02564T for analysis on a polyphasic approach, we describe the characterizations of a novel strain YIM B02564T isolated from this medicinal plant and report that this strain represents a novel species of the genus Neobacillus.

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API ZYM, API 20NE, API 50CH (bioMérieux, France) and Biolog GENIII microplates (BIOLOG Inc., Hayward, CA, United States) were used to view the other biochemical characters and utilization of various substrates according to the manufacturer’s protocols.

Chemotaxonomic characteristics

Several standard methods were applied to analyze the chemotaxonomic characteristics of strain YIM B02564T. Biomass for fatty acid analysis was harvested from cultures grown on TSA medium at 25 °C for 2 days. The fatty acids were extracted using a standard MIDI protocol and identified by using the Sherlock Microbial Identification System (Sherlock version 6.1; MIDI database: TSBA6) following the manufacturer’s instructions (Sasser 2001). Determination of the cell-wall diaminopimelic acid was performed according to the method described by Staneck and Roberts (1974). Menaquinone and polar lipids were extracted following the method described by Minnikin et al. (1984). Menaquinone was analyzed by a reversed-phase HPLC system (Agilent Technologies 1260 Infinity) with a C18 column (25 cm × 4.6 mm, 5 μm). Identification and analysis of polar lipids were performed by a two-dimensional TLC procedure on silica gel G60 plates and then 5% molybdatophosphoric acid, 0.2% ninhydrin and molybdenum blue were used to detect the lipids, aminolipids and phospholipids, respectively.

Results and discussion

Molecular phylogenetic analysis

The complete 16S rRNA gene sequence (1514 bp) was obtained and submitted to GenBank under the accession number MW911620. The 16S rRNA gene sequence similarity values showed that strain YIM B02564T had the highest similarity to Neobacillus fumarioli (97.8%), followed by Neobacillus mesonae (97.4%), Neobacillus soli (97.4%), Neobacillus endophyticus (97.3%) and Neobacillus drentensis (97.3%). The values between YIM B02564T and Neobacillus species were less than the threshold for recognizing a novel species (98.7–99%) (Stackebrandt and Ebers 2006; Kim et al. 2014). The NJ phylogenetic tree based on 16S rRNA gene sequences also showed that strain YIM B02564T was closely related to members of the genus Neobacillus and formed a cluster with N. fumarioli and N. endophyticus (Fig. 1). In conclusion, the phylogenetic analyses based on 16S rRNA gene sequences clearly suggested that the isolate can be considered a species of the genus Neobacillus.

The draft genome of strain YIM B02564T had a total length of 4,629,313 bp with N50 length of 179,987 bp. 12 rRNA genes, 98 tRNA genes and 4449 protein-coding genes were predicted in the annotated result of strain YIM B02564T. The G+C content of strain YIM B02564T calculated from the genome was 41.6 mol%. To confirm the phylogenetic relationship of strain YIM B02564T, a maximum-likelihood (ML) phylogenomic tree was constructed on the basis of 882 orthologous genes. YIM B02564T formed a branch with N. mesonae FJAT-13985T, and formed a broader distinct cluster which also contained N. fumarioli NBRC 102428T and N. endophyticus BRMEA1T (Fig. 2). The dDDH estimate values for YIM B02564T were 21.5% with

![Fig. 1 Phylogenetic tree reconstructed using the neighbor-joining (NJ) algorithm based on the 16S rRNA gene of strain YIM B02564T and closely related strains. Bootstrap values (> 50%) are indicated at the nodes. Scale bar, 0.006 changes per nucleotide position](https://example.com/figure1.png)
129 Page 4 of 7

Archives of Microbiology (2022) 204:129

N. mesonae FJAT-13985T, 18.9% with N. fumarioli NBRC 102428T and 19.8% with N. endophyticus BRMEA1T, which were clearly lower than the recommended cut-off value (70%) (Wayne et al. 1987). Additionally, the ANI values between YIM B02564T and its closely related species N. mesonae, N. fumarioli and N. endophyticus were 77.9%, 76.1% and 75.6%, respectively. AAI values ranged from 63.4 to 77.8% between YIM B02564T and other species in Neobacillus (Table S1). ANI (Richter and Rosselló-Móra 2009) and AAI (Konstantinidis and Tiedje 2005) values were also significantly lower than the threshold of 95–96% for describing prokaryote species. Detailed information for ANI, AAI and dDDH values are given in Table S1. The eleven specific conserved signature indels (CSIs) in protein sequences of strain YIM B02564T were identical with the description of genus Neobacillus (Patel and Gupta 2020). Based on the analysis above, strain YIM B02564T can represent a novel species of the genus Neobacillus.

Phenotypic and chemotaxonomic characteristics

Strain YIM B02564T was found to grow on NA, TSA and R2A medium. Colonies grown on TSA medium were found to be circular, cream-colored and smooth after 3 days of cultivation. Cells of strain YIM B02564T was facultative anaerobic, Gram-positive, oxidase-negative, catalase-positive, rod-shaped (0.4–0.7 µm wide and 2.0–5.0 µm long) and motile with peritrichous flagella (Fig. S1). Ellipsoidal spores were formed at the subterminal position in cells. The optimum growth condition of strain YIM B02564T occurred at 30 °C, pH 7.0 and the peak tolerance to NaCl concentration was 2%. The other results of physiological and biochemical analyses are summarized in the species description and supplementary Table S2, and the properties comparison of strain YIM B02564T with other related species are listed in Table 1.

The cell-wall peptidoglycan of strain YIM B02564T contained glutamic acid, alanine and meso-diaminopimelic acid. The predominant menaquinone was MK-7. The major cellular fatty acids (> 10% of the total fatty acids) were iso-C15:0 (27.6%) and anteiso-C15:0 (23.9%). The minor fatty acids (> 0.5%) were iso-C13:0 (0.6%), iso-C14:0 (5.7%), C14:0 1ω7c alcohol (1.1%), iso-C16:0 (5.2%), C16:1 1ω11c (2.9%), C16:0 (9.4%), iso-C17:1ω10c (0.9%), iso-C17:0 (2.4%), anteiso-C17:0 (5.2%), C17:0 (0.6%), C18:1ω9c (1.4%), C18:0 (0.5%), summed feature 3 (2.3%) and 4 (2.8%). The overall fatty acid profile of strain YIM B02564T was similar to those of the closely related reference type strains, but there were some differences in components (Table S3). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. One unidentified aminophospholipid and four unidentified lipids were also detected (Fig. S2). All these properties are consistent with the general chemotaxonomic features of the genus Neobacillus and support that strain YIM B02564T represents a novel species of this genus, for which the name Neobacillus paridis sp. nov. is proposed.

Description of Neobacillus paridis sp. nov.

Neobacillus paridis (pa’ri.dis. L. gen. n. paridis of Paris, a plant genus, from which the type strain was isolated).

Cells are facultative anaerobic, Gram-positive, oxidase-negative, catalase-positive, rod-shaped, and motile by using...
peritrichous flagella. Ellipsoidal endospores are produced, located subterminally in cells. Colonies on TSA medium at 30 °C are circular, cream-colored and smooth. Growth is achieved between 20 and 55 °C (optimum 30 °C) and with a highest tolerance of 2% NaCl concentration and pH in the range of 6.0–9.0 (optimum 7.0). With the API 20NE test, cells

| Characteristics                        | 1             | 2             | 3             | 4             |
|----------------------------------------|---------------|---------------|---------------|---------------|
| Respiration                            | Facultatively anaerobic | Aerobic | Aerobic | Facultatively anaerobic |
| Cell size (μm)                         | 0.4–0.7×2.0–5.0 | 0.5–0.8×4.0–8.0 | 0.6–1.2×2.2–2.7 | 0.7–1.3×1.5–6.1 |
| Colonies Morphology                    | Circular, cream-coloured, smooth | Irregular, butyrous, brownish-cream, opaque | Pale yellow, flat, opaque, circular/slightly irregular margins | Circular, cream-coloured, smooth, glossy colony |
| Motility                               | Motile | Feebly motile | Motile | Weakly motile |
| pH range for growth (optimum)          | 6.0–9.0 (7.0) | 4.0–6.5 (5.5) | 5.7–9.0 (7.0) | 6.0–8.0 (7.0) |
| Temperature range (optimum, °C)        | 20–55 (30) | 25–55 (50) | 20–45 (30) | 15–50 (25–30) |
| Highest NaCl tolerance (% w/v)         | 2             | 6             | 2             | 0             |
| Nitrate reduction                      | +             | –             | –             | +             |
| β-Galactosidase                        | –             | –             | +             | +             |
| Hydrolysis of aesculin                 | +             | –             | +             | +             |
| API 50CH (acid production)             | –             | v             | –             | –             |
| Glycerol                               | –             | v             | –             | –             |
| D-Ribose                               | –             | v             | +             | –             |
| D-Galactose                            | –             | v             | –             | –             |
| D-Glucose                              | +             | +             | –             | +             |
| D-Fructose                             | –             | +             | –             | +             |
| D-Mannose                              | +             | +             | –             | –             |
| L-Sorbose                              | –             | –             | –             | +             |
| D-Mannitol                             | –             | +             | –             | –             |
| Methyl α-d-glucopyranoside             | –             | v             | –             | –             |
| N-Acetyl glucosamine                   | +             | w             | –             | +             |
| Amygdalin                              | –             | –             | w             | –             |
| Aesculin                               | w             | –             | +             | –             |
| D-Cellobiose                           | –             | –             | +             | –             |
| D-Maltose                              | +             | v             | +             | +             |
| D-Lactose                              | –             | v             | +             | –             |
| D-Melibiose                            | –             | v             | +             | –             |
| D-Sucrose                              | –             | +             | +             | +             |
| D-Trehalose                            | –             | w             | +             | +             |
| D-Melezitose                           | –             | v             | –             | +             |
| D-Raffinose                            | –             | v             | +             | w             |
| Starch                                 | –             | –             | –             | +             |
| Glycogen                               | –             | –             | –             | w             |
| D-Turanose                             | –             | v             | –             | w             |
| G+C content (mol%)                     | 41.6          | 40.4          | 40.3          | 38.5          |

Table 1 Characteristics that differentiate strain YIM B02564T from closely related species of the genus Neobacillus

Taxa: 1, YIM B02564T; 2, N. fumarioli NBRC 102428T (Logan et al. 2000); 3, N. mesonae DSM 25968T (Liu et al. 2014); 4, N. endophyticus BRMEA1T (Jiang et al. 2021). +, Positive; –, negative; w, weakly positive; v, variable

*The DNA G+C contents were calculated based on their genome sequences

are positive for nitrate reduction and hydrolysis of aesculin. API ZYM test show positive reactions for esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphtho-AS-BI-phosphohydrolase and α-glucosidase. API 50CH test indicate that production of acid from α-glucose, β-mannose, N-acetyl glucosamine, α-maltose; and weak production of acid
from aesculin. For the utilization of carbon sources (Biolog GENIII systems), the following substrates are utilized for growth: dextrin, d-maltose, d-trehalose, d-cellobiose, gentiobiose, sucrose, d-turanose, stachyose, d-raffinose, α-d-lactose, d-melibiose, β-methyl-d-glucoside, d-salicin, N-acetyl-β-D-mannosamine, N-acetyl-d-galactosamine, N-acetyl neuraminic acid, α-d-glucose, d-fructose, 3-methyl glucose, N-fucose, L-fucose, L-rhamnose, myo-inositol, glycerol, d-glucose-6-phosphate, l-arginine, l-aspartic acid, l-glutamic acid, d-sorbitol, l-arabitol, pectin, d-galacturonic acid, l-galactonic acid lactone, d-gluconic acid, d-glucuronic acid, gluconamide, mucic acid, quinic acid, p-hydroxy phenylacetic acid. d-Lactic acid methyl ester, l-lactic acid, α-keto-glutaric acid, L-malic acid, bromo-succinic acid, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D, l-butyric acid, α-keto-butyric acid, acetoacetic acid, propionic acid, acetic acid and formic acid. The cell-wall peptidoglycan contains meso-diaminopimelic acid as diagnostic diamino acid. The major menaquinone is MK-7 and the major components in the polar lipid profile are diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl ethanolamine, an unidentified aminophospholipid and four unidentified lipids. The predominant cellular fatty acids are iso-C₁₅:₀ and anteiso-C₁₅:₀.

The type strain is YIM B02564ᵀ (= JCM 34668ᵀ = CGMCC 1.18655ᵀ), isolated from a root of P. polyphylla Smith var. yunnanensis collected from Shilin County, Yunnan Province, southwest China. The GenBank accession numbers for the 16S rRNA gene sequence of Neobacillus paridis YIM B02564ᵀ is MW911620. The whole genome sequences have been deposited at GenBank and GCM Type Strains Genome Database under accession JAESWB000000000 and GCM60020047, respectively.

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Author contributions P-CZ and L-LY analyzed the data and wrote the manuscript. P-CZ, C-JL, ZZ, R-FM, J-RL and X-WJ performed the experiments. L-LY and X-YZ directed the experiment and directed the classification. L-LY takes full responsibility for the final submission. All the authors reviewed and approved the final version of the paper.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication The manuscript is submitted with the consent of all authors.

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