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Bacillus radicibacter sp. nov., a new bacterium isolated from root nodule of *Oxytropis ochrocephala* Bunge

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A Gram-positive, facultative anaerobic, rod-shaped, and endospore-forming strain, designated 53-2^T was isolated from the root nodule of *Oxytropis ochrocephala* Bunge growing on Qilian mountain, China. The strain can grow at pH 7.0–8.0, 10–50 °C and tolerate up to 11% NaCl. Optimal growth occurred at pH 7.2 and 37 °C. The result of BLASTn search based on 16S rRNA gene sequence revealed that strain 53-2^T, being closest related to *Bacillus acidicola* 105-2^T, possessed remote similarity (less than 95.64%) to the species within genus *Bacillus*. The DNA G + C content was 37.8%. Chemotaxonomic data (major quinone is MK-7; major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, unknown phospholipid, and aminoglycophospholipid; fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$ and anteiso-$C_{17:0}$) supported the affiliation of the isolate to the genus *Bacillus*. On the basis of physiological, phylogenetic, and biochemical properties, strain 53-2^T represents a novel species within genus *Bacillus*, for which the name *Bacillus radicibacter* is proposed. The type strain is 53-2^T (=DSM27302^T=ACCC06115^T=CCNWQLS5^T).

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

Genus *Bacillus*, first established by Ferdinand Cohn in 1872, is a large and heterogeneous collection of aerobic or facultative anaerobic rod-shaped, Gram-positive, and endospore-forming bacteria [1]. The type species is *Bacillus subtilis* Cohn 1872-NRS744^T. The genus has developed greatly in the past 30 years and undergone considerable taxonomic changes. At the time of writing this paper, there were 202 species (including seven subspecies) of this genus with validly published names (in spite of the fact that 90 species had been reclassified and transferred into other different genera) (http://www.bacterio.net/bacillus.html).

Oxytropis ochrocephala Bunge, a species within genus *Oxytropis*, is a locoweed that can cause poisoning to animals and distributes mainly in Gansu, Sichuan, and Tsinghai provinces. *O. ochrocephala* Bunge can grow in poor soil and is a dominant legume plant of the Qilian Mountain at the altitude below 3000 m [2]. Investigating the bacteria that inhabit *O. ochrocephala* Bunge can help us understand the plant better and advance in reducing its harm and exploiting economic value. Many rhizobia and bacteria, such as *Sinorhizobium* spp., *Rhizobium* spp., and *Bacillus* spp. were isolated from the root nodules of *O. ochrocephala* Bunge [2, 3]. In this paper, we report the taxonomic characterization of a new bacterium isolated from root nodule of *O. ochrocephala* Bunge.

Materials and methods

Morphological, physiological, and biochemical tests

Strain 53-2^T was isolated and purified as described previously [2]. Routine cultivation was taken out on

Xiu Li Wei and Yan Bing Lin contributed equally to this work.
16S rRNA gene sequence of strain 53-2^T is available under GenBank accession number KC847111. The strain was deposited in DSMZ (Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh) and ACCC (Agricultural Culture Collection of China) under deposition number 27302^T and ACCC 06115^T. All the reference strains were bought from DSMZ.

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Nutrient Agar (NA) (0.5% peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl, and distilled water, pH adjusted to neutral) or in Tryptone Yeast (TY) broth (including 0.5% tryptone, 0.3% yeast extract, 0.07% $CaCl_2.2H_2O$, and distilled water, pH adjusted to neutral) at 37 °C. Unless otherwise indicated, all the morphological, biochemical, and tolerance tests were taken out on NA plate after 48 h incubation.

Colonial morphology was observed after 48 h incubation at 37 °C on NA. For cell morphology observation, the specimen was prepared using the method described previously [4] with a slight modification. First, a drop of distilled water was dripped and a cover slide (diameter $\leq$5 mm) was placed on a colony, keep the slide on for 2–4 h to facilitate cell adhering, and then the slide with cells on was put into 2.5% glutaraldehyde at 4 °C overnight for fixation. The next day, the specimen was rinsed three times using PBS buffer (0.1 M, pH 7.2) for 10 min each to remove excess fixative. Subsequently, the specimen was dehydrated in an ascending ethanol (50, 70, 80, 90, 100%) and 100% acetone for 20 min each. Finally, the sample was rinsed twice in isoamyl acetate (act as dehydrant) for 20 min each. After that, the sample was dried at critical point and coated by platinum and palladium. The specimen was then ready for observation under an S-4800 field emission scanning electron microscopy (FE-SEM, Hitachi) system operating at 15.0 KV.

The motility of strain 53-2^T was examined by inoculating it in semi-solid TY medium using stab method with a straight needle as described by Tittsler and Sandholzer [5].

The forming of endospore was checked by malachite staining and observation under light microscope (Olympus BH-2) as described by Bartholomew and Mittwer [6]. The Gram reaction was examined using the method described by Doetsch [7]. Growth at different temperatures was determined by inoculating strain 53-2^T on NA slant, which was placed in refrigerator (4 °C), water bath (10, 20, 50, 60 °C), and incubator (30, 37 °C). Under different pH values, the growth was examined by inoculating 53-2^T in nutrient broth adjusted to pH 2–12 by KH_2PO_4/HCl, KH_2PO_4/K_2HPO_4, and K_2HPO_4/NaOH buffer systems [8]. At different NaCl concentrations (1–15%, w/v, at 1% intervals), the growth of strain 53-2^T was tested in TY broth supplemented with 1–15 g NaCl per 100 ml. Growth was scored as optical density at 600 nm. Growth in anaerobic environment was assessed at 37 °C on NA plate which was placed in a bag with N_2/CO_2 atmosphere (Genbag anaer, bioMérieux) according to the instruction of the manufacturers.

Catalase activity was evaluated by the production of oxygen bubbles in a 3% (v/v) aqueous hydrogen peroxide solution. The result was positive if there was bubbles appearing in 3 min; otherwise, negative. Oxidase, urease, β-galactosidase and arginine dihydrolase activities, indole production, nitrate reduction, glucose fermentation, and assimilation of 12 carbon sources were determined using API 20NE kits (bioMérieux). Acid production was tested using API 50CH strips (bioMérieux) as recommended by the manufacturers. Utilization of sole carbon sources were determined using Biolog GP2 test system. All the results discriminating 53-2^T from other reference strains are listed in Supplementary Table 1. Cluster analysis was performed by using the free statistical software PAST 3.0 [9, 10]. The results were imported to Past 3.0 software after being standardized (positive, "1"; negative, "0"; weak reaction, blank) to generate a dendrogram using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.

G + C content and16S rRNA gene analysis

Total DNA was prepared using the method described by Marmur [11]. The G + C content of genomic DNA was estimated using thermal denaturation profile according to a formula $(G+C)\% = 51.2 + 2.08 \times [T_m(x) - T_m(k)]$ as described previously [12]. $T_m(x)$ refers to the melting temperature of the tested strain, and $T_m(k)$ refers to that of *Escherichia coli* K12.

16S rRNA gene was amplified using the method previously described by Deng et al. [13], with primers fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rD1 (5′-AAG GAG GTG ATC CAG CC-3′) referring to Weisburg et al. [14]. PCR products were sequenced in Sangon Biotech Co. Shanghai, China. The sequences were used to form contig using DNA Baser software v4.12 (http://www.dnabaser.com), and then the contig-assembled sequence was used in BLASTn search against database EzTaxone [15] to determine closely related species. Due to the fact that genes appearing to be the most similar based on BLASTn hits are not always the closest phylogenetic relatives [16], a phylogenetic analysis is necessary. An alignment was made for 16S rRNA gene sequence of 53-2^T and other available published type strains using Jukes and Cantor model [17]. Phylogenetic tree was constructed using neighbor-joining and maximum-likelihood methods implemented in the software package MEGA version 5.0 [18].

Whole cell fatty acid, quinone, and polar lipid composition

For fatty acid analysis, strain 53-2^T and the four reference strains (*Bacillus acidicola* 105-2^T,*Bacillus kribbensis* BT080^T, *Bacillus vireti* IDA3632^T, and *Bacillus soli* IDA0086^T) were

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cultured on TY agar medium and were harvested after 72 h incubation at 37 °C. Total 60 mg were used to extract fatty acid after being saponified, methylated, and extracted [19, 20]. Fatty acids were analyzed by a Sherlock Microbial Identification System (MIS).

For the determination of respiratory quinones and polar lipids, strain 53-2^T was incubated in TY broth and cultured at 37 °C, the cells were harvested after 72 h and freeze-dried. About 100 mg dry cells were used to extract respiratory quinones. Two-stage method [21, 22] and thin layer chromatography (TLC) were employed for respiratory quinones exaction and purification, respectively. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the TLC plate and further analyzed by a high performance liquid chromatography (HPLC) system (Waters Co.). Another 100 mg dry cells were added into a bottle containing 3.5 ml chloroform–methanol–0.3% aqueous NaCl (50:100:40 by vol) at room temperature to extract polar lipids. After 4 h, the mixture was centrifuged and the supernatant was transferred to a new tube. Another 5 ml chloroform and 0.3% aqueous NaCl were added into the new tube and mixed thoroughly. After phase separation, the aqueous upper layer was discarded and the lower was evaporated to dryness at <37 °C. The dried matter (polar lipids) was dissolved in chloroform–methanol (2:1 by vol) ready for analysis. The lipids were separated by two-dimensional TLC silica gel H plate (100 × 100 mm^2, produced by Qingdao Ocean Chemical Co., Qingdao, China) using method as described by Minnikin et al. [23]. Different reagents for specific polar lipids coloring were used [24].

Results

Morphological, physiological, and biochemical tests

Cells morphology of strain 53-2^T were shown in Supporting Information Fig. S1. An ellipsoidal endospore with slightly swollen sporangia is clearly visible in the central or subterminal of the cell. The growth conditions were given in species description.

There was only a clean growth line along the inoculation trace without diffuse zone spreading in the motility test. The result indicated the strain was non-motile.

The result of test using the Biolog GP2 microplate revealed no strain in database showing more than 50% similarity with strain 53-2^T which possessed a very narrow spectrum of substrate utilization. There were only four substrates that were utilized by 53-2^T in GP2 microplate. *Bacillus kribbensis* BT080^T and *Bacillus soli* IDA0086^T also possessed a narrow spectrum but of different substrates to 53-2^T (details available in Supporting Information Table S1). *Bacillus acidicola* 105-2^T and *Bacillus vireti* IDA3632^T possessed relatively wider-spectrum of substrates utilization than strain 53-2^T. All the results of Biolog GP2, API 50CHB, API 20NE, and API EYM that differentiated 53-2^T from reference strains were given in Supporting Information Table S1. Sometimes, varied results with the same substrate in GP2 (Biolog), API 20NE, and API 50CHB (bioMérieux) tests were obtained. For example, negative result of D-fructose utilization in test using GP2 microplate was detected, but it was positive in test using 50CHB (details available in Supporting Information Tables S1 and 2). The dendrogram, based on the phenotypic characteristics (obtained from API and Biolog GP2 tests), is showed in Fig. 1, displaying strain 53-2^T clustered together with the other four reference strains only at less than 32% similarity.

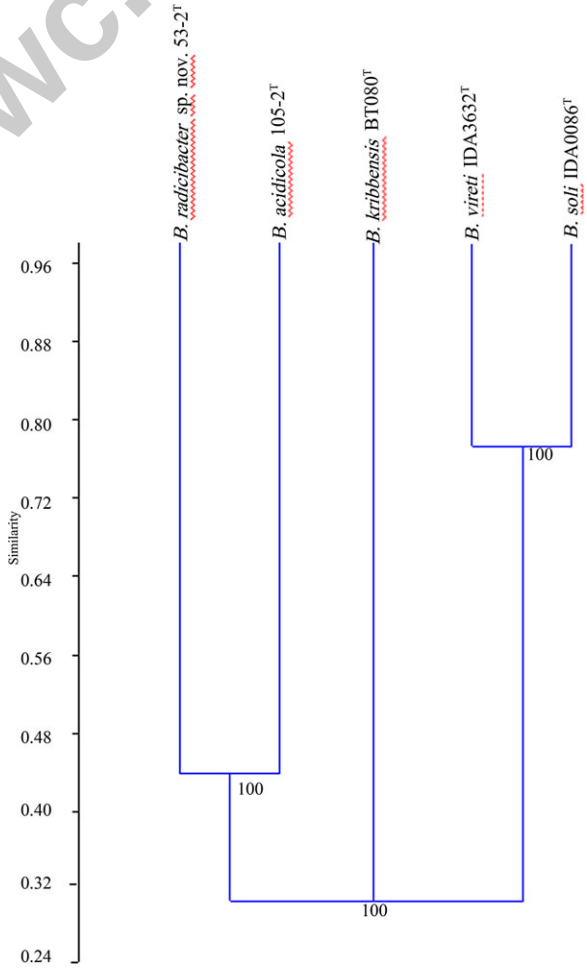

Figure 1. Dendrogram based on phenotypic characterizatics displaying the similarity of 53-2^T and four reference strains. The dendrogram was derived from euclidean distances. Numbers at branching points referring to bootstrap values (1000 resampling).

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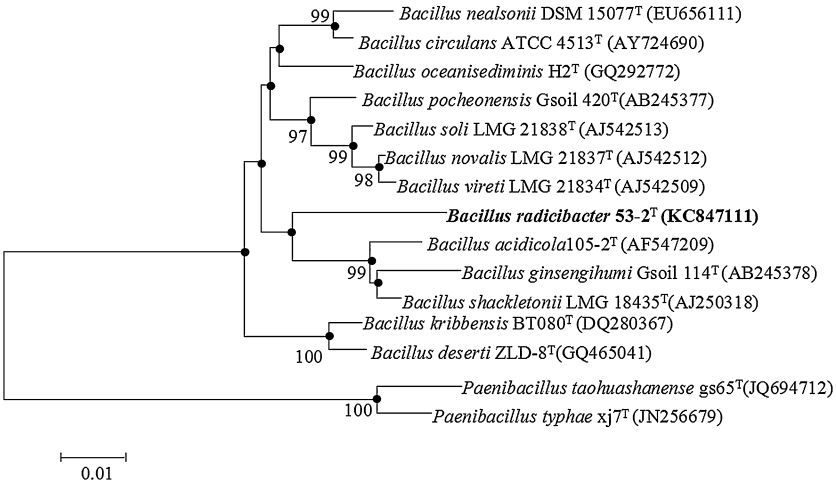

Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences displaying the relationships between 53-2^T and 12 type species within genus *Bacillus*. *Paenibacillus typhae* xj7^T and *Paenibacillus taohuashanense* gs 65^T were used as outgroup. Numbers at branching points referring to bootstrap values (1000 resampling). Evolutionary distances were calculated using Jukes–Cantor model. Scale bar: 0.01 changes per nucleotide position. The filled circles indicate the corresponding nodes also found in the tree generated by maximum likelihood method.

The closest related species was *Bacillus acidicola* 105-2^T which formed a branch with 53-2^T at less than 48% similarity level with 100% bootstrap value.

G + C content and16S rRNA gene analysis

In this study, the DNA G + C content of strain 53-2^T showed out to be 37.8% and other closely related *Bacillus* species range from 39.8 to 43.3% (Supporting Information Table S1).

In the phylogenetic tree generated by neighbor-joining method based on 16S rRNA gene sequences (Fig. 2), strain 53-2^T clustered together with *Bacillus acidicola* 105-2^T and two other *Bacillus* species, being closest related to *Bacillus acidicola* 105-2^T with 95.6% similarity. *Bacillus kribbensis* BT080^T, *Bacillus vireti* IDA3632^T, and *Bacillus soli* IDA0086^T located in the neighborhood of 53-2^T. The same topologies were also found in phylogenetic tree generated by maximum likelihood (data not shown) and the corresponding nods were indicated by filled small black circles in Fig. 2.

Whole cell fatty acid, quinone, and polar lipid composition

The major fatty acids of strain 53-2^T were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ (17.4%), and iso-$C_{15:0}$ (details are given in Table 1), all of which also existed in the reference strains but with different percentages. *Bacillus acidicola* 105-2^T possessed the same major fatty acids with strain 53-2^T.

The major quinone of 53-2^T turned out to be MK-7, which was consistent with majority of species in genus *Bacillus* and supported affiliation of 53-2^T to the genus *Bacillus* [25–27].

The result of polar lipids test revealed that strain 53-2^T contained diphosphatidylglycerol, phosphatidyglycerol, unidentified phospholipid, and two unknown amino-glycophospholipids (details available in Supporting Information Fig. S2). The four lipids were present with almost equivalent amounts.

Table 1. Fatty acids of strain 53-2^T and closely related phylogenetic neighbors.

	Content (%) in strain				
Fatty acid	**1**	**2**	**3**	**4**	**5**
10:0	/	/	/	/	1.3
11:0 anteiso	/	/	/	1.8	/
14:0	/	/	1.2	/	/
14:0 iso	5.0	1.6	2.2	14.3	/
15:0 anteiso	50.6	42.2	71.0	35.5	39.0
15:0 iso	9.5	24.2	3.9	17.2	27.2
16:0	/	/	2.3	1.7	1,9
16:0 iso	6.7	3.0	2.0	13.6	2.9
16:1ω7*c* alcohol	6.1	1.1	/	2.8	2.9
16:1ω11*c*	/	/	1.0	1.2	2.0
17:1 iso ω10*c*	/	/	/	/	3.2
17:0 anteiso	17.4	21.2	10.8	5.1	2.5
17:0 iso	/	3.2	/	/	2.1
summed feature 4	4.7	3.6	/	/	2.1

Strains: 1, *B. radicibacter* sp. nov. 53-2T; 2, *B. acidicola* 105-2T; 3, *B. kribbensis* BT080T; 4, *B. vireti* IDA 3632T; 5, *B. soli* IDA0086T. Values are percentages of total fatty acids and were obtained in this study; fatty acids accounting for <1.0% of the total content in all strains are omitted. Summed feature represents group of two or three fatty acids that could not be separated by gas chromatography with the Sherlock Microbial Identification System (MIDI). Summed feature 4 contains 17:1 iso I/anteiso B, and/or 17:1 anteiso B/iso I. "/" represents not detected.

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Discussion

Strain 53-2^T was Gram positive, rod-shaped, endospore-forming, and aerobic. According to the Gram positive rods determination in Bergey's Manual of Determinative Bacteriology [28], we can deduce that the strain belongs to *Bacillus* spp., and the BLASTn search based on 16S rRNA gene sequence supported this point.

The negative result in motility test was in consistent with the fact that strain 53-2^T has no flagellum when observed under FE-SEM. The two results corroborated each other. The other four reference strains are all motile, so strain 53-2^T can be readily differentiated by non-motility.

Varied reaction of the same substrate in different testing systems had been reported previously [29] and detected in our lab by other people (data not shown). That may be caused by the different evaluation of the API 50CHB test (based on acid production) and the Biolog GP2 test (based on proprietary tetrazolium redox activities), composition of the inoculm solution, or substrate concentrations [29, 30]. The bootstrap values in dendrogram based on phenotypical characteristics (obtained from API and Biolog GP2 tests) are all 100% revealed that the nodes received 100% support, that is to say the dendrogram is stable and believable and can be used for further analysis. Strain 53-2^T clustered with the four type strains at no more than 32% similarity, being closest to *Bacillus acidicola* 105-2^T at no more than 48% similarity. The result confirmed that 53-2^T is a new member within genus *Bacillus*. Hydrolyses of tween 40, tween 80, and adenosine, and producing acid from potassium 2-ketogluconate are four most efficient characters that distinguish 53-2^T from the four types strains.

Generally, the G + C% range observed is no more than 10% within a well-defined genus [31]. G + C% of strain 53-2^T (37.8%) is only a little lower than the four type strains (39.8–43.3%), supporting 53-2^T and belongs to the same genus with the four reference strains. The phylogenetic analysis based on 16S rRNA gene sequences supported the point and revealed that strain 53-2^T should be considered as a new member in *Bacillus* genus according to the low similarity (95.64%) [32].

In fatty acid analysis, the result revealed anteiso-$C_{15:0}$ was the largest component in all strains, while the published data indicated iso-$C_{15:0}$ was the largest except in *Bacillus kribbensis* BT080^T [25, 26, 33]. We think the divergence was caused by the usage of different media for cultivation.

The presence of the unknown phospholipid and the absence of phosphatidylethanolamine distinguished strain 53-2^T from *Bacillus acidicola* 105-2^T and *Bacillus kribbensis* BT080^T [25, 26]. The small quantity of aminoglycophospholipid might be phosphatidylserine which has been reported in *Bacillus isronensis* [34].

Based on all the taxonomic data described above, strain 53-2^T represents a novel species within genus *Bacillus*, for which the name *Bacillus radicibacter* sp. nov. is proposed.

Description of *Bacillus radibacter* sp. nov.

Bacillus radicibacter sp. nov. (ra.di.ci.bac'ter. L. fem. n. *radix*, *radicis*, root; N.L.masc. n. *bacter*, rod; N.L. masc. n. *radicibacter* referring to a rod-shaped organism originating from a root nodule of *Oxytropis ochrocephala* Bunge).

The cells of strains 53-2^T are rod-shaped, 1.3–2.7 μm in length and 0.5–0.8 μm wide. Colonies on NA after 48 h incubation at 37 °C are 0.4–2.0 mm in diameter. Colonies are smooth, cream-white, and circular. The temperature and pH ranges for growth are 10–50 °C and 7.0–8.0, respectively. Cells are facultative anaerobic, Gram- and catalase-positive, urease- and oxidase-negative. Cells are non-motile without flagella. Cells are tolerant of up to 11% (w/v) NaCl in TY broth. Ellipsoidal endospore with swollen sporangia can occur in the subterminal or central of cell. The cells can reduce nitrate to nitrites, hydrolyze esculin and gelatin, cannot produce indole from tryptophan or ferment glucose. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, unidentified phospholipid, and aminoglycophospholipid. MK-7 is the primary quinone of 53-2^T. The DNA G + C content for strain 53-2^T is 37.8%. The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, and anteiso-$C_{17:0}$.

The cells of strain 53-2^T grow well in tween 40, tween 80, α-ketovaleric acid, and glycerol; weakly in β-cyclodextrin, dextrin, D-cellobiose, D-gluconic acid, β-methyl-D-glucoside, pyruvic acid, and adenosine of Biolog microplate. When tested using API 20NE strip, the strain assimilates D-glucose, *N*-acetyl-L-glucosamine, D-maltose, and potassium gluconate. In the test using API 50CHB strip, the cells produce acid from glycerol, D-glucose, D-fructose, *N*-acetylglucosamine, esculin ferric citrate, D-cellobiose, D-maltose, D-saccharose, potassium 2-ketogluconate, and potassium 5-ketogluconate. When tested with API ZYM strip, strain 53-2^T possessed activities of alkaline phosphatase, α-glucosidase, and weakly of esterase, esterase lipase, cystine arylamidase, Naphthol-AS-Bl- phosphohydrolase. Other tests with GP2 microplate and API kits (API 20NE, API ZYM, API 50CHB) not mentioned here are all of negative results.

The bacterium was isolated from root nodule of *Oxytropis ochrocephala* growing on Qilian mountain, China

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(GPS location of sampling site is N38°01′45″, E101° 25′57″). The type strain is 53-2^T (=DSM27302^T=ACCC06115^T=CCNWQLS5^T).

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