Paenibacillus jilunlii sp. nov., a nitrogen-fixing species isolated from the rhizosphere of *Begonia semperflorens*

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A nitrogen-fixing bacterium, designated strain Be17^T, was isolated from rhizosphere soil of *Begonia semperflorens* planted in Beijing Botanical Garden, PR China. Phylogenetic analyses based on a segment of the *nifH* gene sequence and a full-length 16S rRNA gene sequence revealed that strain Be17^T was a member of the genus *Paenibacillus*. High levels of 16S rRNA gene sequence similarity were found between strain Be17^T and *Paenibacillus graminis* RSA19^T (97.9%), *Paenibacillus sonchi* LMG 24727^T (97.8%), *Paenibacillus riograndensis* CECT 7330^T (96.2%) and *Paenibacillus borealis* DSM 13188^T (96.1%), respectively. Levels of 16S rRNA gene sequence similarity between strain Be17^T and the type strains of other recognized members of the genus *Paenibacillus* were below 96.0%. However, the DNA–DNA hybridization values between strain Be17^T and *P. graminis* RSA19^T, *P. sonchi* LMG 24727^T and *P. riograndensis* CECT 7330^T were 47.9%, 38.7% and 37.5%, respectively. The DNA G+C content of strain Be17^T was 52.9 mol%. The major fatty acid component of strain Be17^T was anteiso-branched C_{15:0} (30.92%). The major isoprenoid quinone was MK-7. The cell-wall peptidoglycan contained meso-diaminopimelic acid as the diagnostic diamino acid. On the basis of its phenotypic characteristics, 16S rRNA gene sequences, DNA G+C content, DNA–DNA relatedness, chemotaxonomic properties and *nifH* gene sequence, strain Be17^T represents a nitrogen-fixing strain of a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus jilunlii* sp. nov. is proposed. The type strain is Be17^T (=CGMCC 1.10239^T=DSM 23019^T).

As early as 1908, nitrogen-fixing strains of *Bacillus polymyxa* were found to be associated with plants (Von Bredemann, 1908). In 1991, Ash *et al.* (1991) proposed the splitting of the genus *Bacillus* into three distinct groups. The genus *Paenibacillus* was then erected (Ash *et al.*, 1993, 1994) on the basis of phylogenetic and 16S rRNA gene sequence analyses of group 3 bacilli. Continued transfers of species of the genus *Bacillus* to the genus *Paenibacillus* and further descriptions of novel members of the genus *Paenibacillus* have increased the number of species in the genus *Paenibacillus* considerably (Shida *et al.*, 1997; Saha *et al.*, 2005). At the time of writing, the genus contained more than 100 species (<http://www.bacterio.cict.fr/p/paenibacillus.html>) and the type strains of 16 species

have been reported to exhibit nitrogenase activities, including *Paenibacillus polymyxa*, *P. macerans*, *P. peoriae*, *P. graminis*, *P. odorifer*, *P. brasiliensis*, *P. azotofixans*, *P. borealis*, *P. wynnii*, *P. massiliensis*, *P. sabinae*, *P. zanthoxyl*, *P. donghaensis*, *P. forsythiae*, *P. riograndensis* and *P. sonchi* (Beneduzi *et al.*, 2010; Coelho *et al.*, 2009; Hong *et al.*, 2009).

Members of this genus are widely distributed in nature and have diverse physiological characteristics. Some species consistently show a high capacity to fix atmospheric nitrogen *in vitro* (Elo *et al.*, 2001; Berge *et al.*, 2002; Ma *et al.*, 2007; Ma & Chen, 2008). In this study, we describe the isolation and classification of a novel nitrogen-fixing species within the genus *Paenibacillus* based on phenotypic properties, 16S rRNA gene sequences, DNA G+C content, DNA–DNA relatedness, chemotaxonomic properties and *nifH* gene sequences.

The bacterial strain studied was isolated from rhizosphere soil of *Begonia semperflorens* plants planted in Beijing

Abbreviation: SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of *Paenibacillus jilunlii* sp. nov. Be17^T are GQ985393 and GQ985392, respectively.

Supplementary figures are available with the online version of this paper.

Botanical Garden, Beijing, PR China. The soil was collected near the plant roots from a depth of 30 cm. Samples were transported to the laboratory as soon as possible. A 1 g sample of soil was placed in 9 ml sterile water and stirred for 30 min. Aliquots of 100 µl from the aqueous portion of the mixture were heated at 80 °C for 15 min and then spread on nitrogen-free medium (Ma *et al.*, 2007) in triplicate and incubated at 30 °C. One colony that grew very well on nitrogen-free medium and exhibited nitrogenase activity was selected and incubated at 30 °C for 3 days for further study.

To determine cell morphology, the novel isolate, strain Be17^T, was grown on endospore-forming medium (0.07 % yeast extract, 0.1 % tryptone, 0.1 % glucose, 0.02 % (NH₄)₂SO₄, 0.02 % MgSO₄·7H₂O, 0.1 % K₂HPO₄ (w/v), pH 7.0) for 14 h. Cell morphology was examined by scanning electron microscopy (SEM). Cell motility was determined by observing the growth spread of cells in test tubes containing semi-solid Lombard–Dowell (LD) medium after incubation for 3 days. To determine whether the novel strain exhibited aerotactic activity, bacterial cells were incubated by mixing with semi-solid LD medium at 40–50 °C in test tubes followed by incubation at 30 °C for 4 days.

Chromosomal DNA was extracted and purified according to Sambrook *et al.* (1989) with some modifications. A nearly complete sequence of the 16S rRNA gene was obtained from a PCR product amplified from strain Be17^T by using the universal forward primer P1 and the universal reverse primer P6. Primer P1 (5'-AGAGTTTGATCCTG-GTCAGAACGCT-3') corresponded to positions 8–37 and primer P6 (5'-TACGGCTACCTTGTTACGACTTCACCC-C-3') corresponded to positions 1479–1506 in the *Escherichia coli* 16S rRNA gene (Yanagi & Yamasato, 1993) and the fragment was sequenced as described by Ding *et al.* (2005). The 16S rRNA gene sequence was analysed using the EzTaxon server (Chun *et al.*, 2007). The evolutionary distance was calculated and the phylogenetic trees were constructed using the MEGA3.1 software package (Kumar *et al.*, 2004) with neighbour-joining and maximum-likelihood methods.

DNA–DNA hybridization and DNA G+C content were determined as described by Ziemke *et al.* (1998) and Tamaoka & Komagata (1984), respectively.

Strains belonging to the genus *Paenibacillus* are often identified by PCR amplification of a 16S rRNA gene fragment with the highly specific forward primer PAEN515F (5'-GCTCGGAGAGTGACGGTACCTGAGA-3') (Shida *et al.*, 1997) and the universal reverse primer 1377R (5'-GGCA-TGCTGATCCGCGATTACTAGC-3') (Shida *et al.*, 1996). In this study, the novel isolate was further identified by this PCR amplification system.

To confirm the nitrogen-fixing capacity of strain Be17^T, an assay for nitrogenase activity was performed and PCR amplification of the *nifH* gene was conducted. The *nifH*

gene PCR amplification was performed as described by Ding *et al.* (2005). For measurement of nitrogenase activity, strain Be17^T and several reference strains were analysed by the acetylene reduction assay method (Berge *et al.*, 2002).

Most physiological and biochemical tests, including Gram staining and determination of the optimal temperature and pH for growth, were performed using conventional methods. Catalase activity was analysed by bubble formation in a 3 % (v/v) H₂O₂ solution. Hydrolysis of casein and starch was determined as described by Cowan & Steel (1965). The utilization of various substrates as carbon and energy sources was determined according to Shirling & Gottlieb (1966). Resistance to antibiotics was tested as described by Gao *et al.* (1994). Tests for nitrate reduction, production of dextrin, the Voges–Proskauer reaction and growth inhibition by NaCl and lysozyme were performed according to Gordon *et al.* (1973), Priest *et al.* (1981) and Rhodes-Roberts (1981), respectively.

Analysis of cellular fatty acid content was conducted as described by Komagata & Suzuki (1987) using the Sherlock Identification System (MIDI) (Sasser *et al.*, 2005). Cellular menaquinones were extracted and purified as described by Collins (1985) and then analysed by HPLC. Polar lipids were extracted according to the method of Minnikin *et al.* (1979) and were identified by two-dimensional TLC and spraying with specific reagents as described by Collins *et al.* (1980). The isomer type of the diamino acid of the cell-wall peptidoglycan was determined using the methods described by Schleifer & Kandler (1972) and Komagata & Suzuki (1987).

Cells of strain Be17^T examined by SEM (see Supplementary Fig. S1 in IJSEM Online) showed that ellipsoidal spores were located subterminally in swollen sporangia. From the growth spread morphology and the observation that the clone grew as well on the surface as it did on the bottom of the semi-solid medium test tube, it was concluded that cells of strain Be17^T were motile and facultatively anaerobic.

The nearly complete 16S rRNA gene sequence (1467 nt) of strain Be17^T was compared by BLAST analysis with the sequences held in GenBank/EMBL/DDBJ. This analysis indicated that the novel strain was phylogenetically related to members of the genus *Paenibacillus*. Phylogenetic trees showing the position of the novel strain in relation to other species of the genus *Paenibacillus* are shown in Fig. 1 and Supplementary Fig. S2 (available with IJSEM Online). No significant differences were found between the phylogenetic trees obtained by the two different treeing methods. A more detailed phylogenetic tree that includes additional recognized species of the genus *Paenibacillus* is available as Supplementary Fig. S3. Strain Be17^T showed high levels of 16S rRNA gene sequence similarity with *P. graminis* RSA19^T (97.9 %), *P. sonchi* LMG 24727^T (97.8 %), *P. riograndensis* CECT 7330^T (96.2 %) and *P. borealis* DSM 13188^T (96.1 %). Levels of 16S rRNA gene sequence similarity between strain Be17^T and the type strains of

Fig. 1. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences showing the position of strain Be17^T among species of the genus *Paenibacillus*. *Bacillus subtilis* NCDO 1769^T was used as an outgroup. Bootstrap analyses were performed with 1000 replications. Bar, 20 substitutions per nucleotide position.

other recognized members of the genus *Paenibacillus* were below 96.0%. After amplification by PCR with PAEN515F and 1377R as the forward and reverse primers, respectively, specific segments of about 900 bp were obtained from strain Be17^T. This result further demonstrated that the novel isolate was a member of the genus *Paenibacillus*.

DNA–DNA hybridization tests were performed to determine the genomic relatedness between the novel strain and the type strains of *P. graminis*, *P. sonchi* and *P. riograndensis*. The DNA–DNA hybridization values between strain Be17^T and *P. graminis* RSA19^T, *P. sonchi* LMG 24727^T and *P. riograndensis* CECT 7330^T were 47.9%, 38.7% and 37.5%, respectively. According to the recommendations of the ad hoc committee, strain Be17^T and the three type strains did not belong to the same species, as the value found for DNA–DNA relatedness was lower than the threshold value of 70% (Wayne *et al.*, 1987). The DNA G+C content of strain Be17^T was 52.9 mol% (Table 1), which was within the range of values expected for members of the genus *Paenibacillus* (Shida *et al.*, 1997).

A 325 bp segment of the *nifH* gene was amplified by using two degenerate primers for the nitrogenase Fe protein gene. Phylogenetic trees were generated by the neighbour-joining and maximum-parsimony methods by using the MEGA3.1 software package. Phylogenetic analysis based on *nifH* gene sequences revealed that strain Be17^T clustered together with species of the genus *Paenibacillus* (Fig. 2 and Supplementary Fig. S4). The *nifH* gene sequence similarity between strain Be17^T and *P. sonchi* LMG 24727^T, *P. borealis* DSM 13188^T, *P. forsythiae* DSM 17842^T, *P. odorifer* TOD45^T and *P. wynnii* LMG 22176^T was 95.4%, 82.6%, 79.6%, 77.5% and 77.4%, respectively. The results of the nitrogenase assay, as shown in Table 2, showed that strain Be17^T exhibited significant nitrogenase activity.

Colonies of strain Be17^T on LD medium were circular, convex, yellow and glossy with entire margins. The phenotypic characteristics of strain Be17^T and several

Table 1. Differential phenotypic characteristics between strain Be17^T and the type strains of selected members of the genus *Paenibacillus*

Taxa: 1, strain Be17^T; 2, *P. sonchi* LMG 24727^T; 3, *P. riograndensis* CECT 7330^T; 4, *P. graminis* DSM 15220^T; 5, *P. stellifer* DSM 14472^T; 6, *P. sabiniae* DSM 17841^T. +, Positive reaction; –, negative reaction.

Characteristic	1	2	3	4	5	6
Optimum growth temp. (°C)	30	30	30	30	28	30
3% (w/v) NaCl	+	+	+	+	–	+
Growth with lysozyme (0.001%)	+	+	+	–	–	–
Voges–Proskauer	–	+	+	–	–	+
Nitrate reduction	+	–	–	+	–	+
Production of dextrin	–	+	–	–	+	–
Hydrolysis of:						
Casein	–	+	–	–	–	–
Starch	+	–	+	–	+	–
Acid production from:						
L-Aspartate	–	–	–	+	–	–
D-Fructose	+	+	+	–	+	–
D-Glucose	+	–	+	+	+	–
Glycerol	+	–	+	+	–	–
Lactose	+	–	+	+	+	–
Maltose	+	–	+	+	+	–
Sodium citrate	+	+	–	–	–	–
D-Sorbitol	–	–	+	–	–	–
Sucrose	+	–	+	–	+	–
D-Xylose	+	–	+	+	+	–
DNA G+C content (mol%)	52.9	46.8	55.1	52.1	55.6*	51.8

*Data from Suominen *et al.* (2003).

Fig. 2. Maximum-parsimony phylogenetic tree based on partial *nifH* gene sequence comparisons, showing the position of strain Be17^T. *Mesorhizobium ciceri* was used as the outgroup. Bootstrap analyses were performed with 1000 replications. Only bootstrap values >50% are shown at the branch points. Bar, 20 substitutions per nucleotide position.

closely related species of the genus *Paenibacillus* are presented in Table 1. Strain Be17^T was found to have distinct physiological properties (Table 1) that enabled it to be distinguished from these recognized species of the genus *Paenibacillus*.

The major fatty acid profiles of strain Be17^T and the type strains of closely related members of the genus *Paenibacillus* are shown in Table 3. The major fatty acid usually found in recognized members of the genus *Paenibacillus*, anteiso-C_{15:0}, was also the major fatty acid component of strain Be17^T (30.92%). The presence of MK-7 as the major menaquinone component was in agreement with the description of the genus *Paenibacillus*. The major polar lipid found for strain Be17^T was

diphosphatidylglycerol. Traces of phosphatidylmethylethanolamine, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylglycerol and lysophosphatidylglycerol were also detected (see Supplementary Fig. S5 in IJSEM Online). The novel strain contained meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan.

In summary, phylogenetic analysis based on the full-length 16S rRNA gene sequence, a fragment of the *nifH* gene sequence, DNA G+C content and chemotaxonomic properties revealed that strain Be17^T was a member of the genus *Paenibacillus*. The phenotypic characteristics and low levels of DNA–DNA relatedness (below 70%) further demonstrated that the strain Be17^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus jilunlii* sp. nov. is proposed.

Description of *Paenibacillus jilunlii* sp. nov.

Paenibacillus jilunlii (ji.lun'li.i. N.L. masc. gen. n. *jilunlii* of Jilun Li, named after Professor Jilun Li, one of the academicians of the Chinese Academy of Sciences and a pioneer of microbiology in PR China).

Cells stain Gram-positive and are facultatively anaerobic, motile, straight rod-shaped and measure 1.0–1.5 µm × 2.5–3.5 µm. Ellipsoidal spores are located subterminally in swollen sporangia. Colonies on LD medium are circular, convex, yellow and glossy with entire margins after 72 h incubation at 30 °C and are usually 1.5–2.5 mm in diameter. The temperature range for growth is 15–50 °C, with optimal growth at 30 °C. The pH range for growth is 4–9; optimal pH is 6.0. Grows at 3% (w/v) NaCl, but is

Table 2. Nitrogenase activity of strain Be17^T compared with some other nitrogen-fixing species of the genus *Paenibacillus*

Results are means ± SD of three determinations.

Strain	Nitrogenase activity [nmol C ₂ H ₄ (mg protein h) ⁻¹]
<i>P. zanthoxyli</i> DSM 18202 ^T	4326 ± 127.8
<i>P. durus</i> ATCC 35681	1623 ± 89.4
<i>P. sabinae</i> DSM 17841 ^T	287 ± 73.5
<i>P. forsythiae</i> DSM 17842 ^T	268 ± 55.4
<i>P. odorifer</i> TOD45 ^T	13.4 ± 1.33
<i>P. polymyxa</i> ATCC 842 ^T	8.74 ± 0.84
<i>P. graminis</i> RSA19 ^T	183.5 ± 33.7
Be17 ^T	236.86 ± 36.7

Table 3. Cellular fatty acid profiles of strain Be17^T and closely related type strains of the genus *Paenibacillus*

Taxa: 1, strain Be17^T; 2, *P. sonchi* LMG 24727^T; 3, *P. riograndensis* CECT 7330^T; 4, *P. graminis* DSM 15220^T; 5, *P. durus* DSM 1735^T; 6, *P. zanthoxyli* DSM 18202^T; 7, *P. forsythiae* DSM 17842^T; 8, *P. sabinae* DSM 17841^T. Values are percentages of the total fatty acids. For unsaturated fatty acids, the position of the double bond can be located by counting from the methyl (ω) end of the carbon chain; *cis* isomers are indicated by the suffix *c*. The results shown were determined under the same conditions for all strains. ND, not detected.

Fatty acid	1	2	3	4	5	6	7	8
Saturated straight-chain								
C _{14:0}	5.22	3.61	6.96	7.35	4.76	4.84	4.79	2.93
C _{16:0}	11.78	8.58	16.78	7.06	19.87	12.88	18.59	18.07
C _{18:0}	ND	0.91	0.49	2.43	5.48	6.77	5.73	3.24
Saturated iso-branched								
iso-C _{14:0}	15.03	22.12	4.97	28.48	5.31	3.79	2.66	3.24
iso-C _{15:0}	4.30	4.71	6.29	4.97	7.89	1.63	0.99	1.40
iso-C _{16:0}	13.32	16.10	6.53	11.18	10.58	14.90	11.53	14.44
iso-C _{17:0}	ND	0.64	0.89	ND	1.48	0.59	0.95	0.82
Saturated anteiso-branched								
anteiso-C _{15:0}	30.92	35.95	44.45	36.23	39.76	32.25	29.97	36.62
anteiso-C _{17:0}	1.88	1.07	1.79	0.37	1.94	3.87	4.61	6.34
Unsaturated								
C _{18:1ω9c}	ND	0.37	0.85	0.61	2.12	1.54	4.83	3.85

unable to tolerate 5 % (w/v) NaCl. Growth is not inhibited by 0.001 % (w/v) lysozyme. Catalase- and oxidase-negative. Negative for the Voges–Proskauer reaction. Nitrate is reduced to nitrite. Nitrogen is fixed. The following substrates are utilized for growth and acid production: cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, raffinose, trehalose, D-xylose, inositol, inulin, glycerol, lactose, L-arabinose, maltose, mannitol, N-acetyl-D-glucosamine, sodium citrate and sucrose. Acids are not produced from creatine, D-sorbitol, DL-malic acid, L-sorbose, L-aspartate, sodium DL-malate or sodium succinate. Gelatin is not hydrolysed. Starch is hydrolysed, but casein is not. The predominant menaquinone is MK-7. The cell wall contains *meso*-diaminopimelic acid as the diagnostic diamino acid. The major fatty acids are anteiso-C_{15:0} and iso-C_{14:0}. The type strain is sensitive to ($\mu\text{g ml}^{-1}$): ampicillin (100), gentamicin (40) and chloramphenicol (30), but resistant to kanamycin (50), spectinomycin (100), streptomycin (40) and tetracycline (12.5).

The type strain, Be17^T (=CGMCC 1.10239^T=DSM 23019^T), was isolated from the rhizosphere soil of *Begonia semperflorens* grown in Beijing, P. R. China. The DNA G+C content of the type strain is 52.9 mol% (thermal denaturation method).

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