Paenibacillus sophorae sp. nov., a nitrogen-fixing species isolated from the rhizosphere of *Sophora japonica*

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A nitrogen-fixing bacterium, designated strain S27^T, was isolated from rhizosphere soil of *Sophora japonica*. Phylogenetic analysis based on a fragment of the *nifH* gene and the full-length 16S rRNA gene sequence revealed that strain S27^T is a member of the genus *Paenibacillus*. High levels of 16S rRNA gene sequence similarity were found between strain S27^T and *Paenibacillus durus* DSM 1735^T (97.3%), *Paenibacillus sabinae* DSM 17841^T (96.9%), *Paenibacillus forsythiae* DSM 17842^T (96.7%) and *Paenibacillus zanthoxyli* DSM 18202^T (96.6%). However, DNA–DNA hybridization values between strain S27^T and the four type strains were 37.64%, 23.12%, 25.6% and 34.99%, respectively. Levels of 16S rRNA gene sequence similarity between strain S27^T and the type strains of other recognized members of the genus *Paenibacillus* were below 96.5%. The DNA G + C content of strain S27^T was 46.0 mol%. The major fatty acids were anteiso-C_{15:0}, C_{16:0} and iso-C_{16:0}. The major isoprenoid quinone was MK-7. On the basis of its phenotypic characteristics and DNA–DNA hybridization results, strain S27^T is considered to represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus sophorae* sp. nov. is proposed. The type strain is S27^T (=CGMCC 1.10238^T =DSM 23020^T).

The genus *Paenibacillus* was described by Ash *et al.* (1993) on the basis of analysis of the 16S rRNA gene sequences of group 3 bacilli. Members of the genus are widely distributed in nature and have diverse physiological characteristics. Some species consistently show a great capacity to fix atmospheric nitrogen *in vitro* (Elo *et al.*, 2001; Berge *et al.*, 2002; Ding *et al.*, 2005; Ma *et al.*, 2007a, b). At the time of writing, there are more than 100 species in this genus, including 16 species having nitrogen-fixing ability. These are *Paenibacillus polymyxa*, *P. macerans*, *P. peoriae*, *P. graminis*, *P. odorifer*, *P. brasiliensis*, *P. azotofixans*, *P. borealis*, *P. wynnii*, *P. massiliensis*, *P. sabinae*, *P. zanthoxyli*, '*P. donghaensis*', *P. forsythiae*, *P. riograndensis* and *P. sonchi* (Beneduzi *et al.*, 2010; Coelho *et al.*, 2009; Hong *et al.*, 2009). Here we show that a bacterial strain, designated S27^T, isolated from rhizosphere soil of *Sophora japonica* represents a novel nitrogen-fixing species of the genus *Paenibacillus*.

Strain S27^T was isolated from rhizosphere soil of *Sophora japonica* from Fenghuang mountain (39° 54' N 116° 23' E) in the north-west of Beijing, People's Republic of China.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of strain S27^T are GQ985395 and GQ985394, respectively.

Five supplementary figures are available with the online version of this paper.

Soil was collected at a depth of 30 cm below ground near the plant root then transported to the lab as soon as possible. The soil sample was placed in 9 ml sterile water and stirred for 30 min. The aqueous portion (100 µl) was heated at 80 °C for 15 min and then spread on nitrogen-free medium (Ma *et al.*, 2007a, b) in triplicate and incubated at 30 °C. After 3 days of incubation, strain S27^T was selected for further study. Strain S27^T was the only isolate with nitrogen-fixing ability found in this habitat.

To confirm the nitrogen-fixing capacity of strain S27^T, an assay for nitrogenase activity and PCR amplification of the *nifH* gene were carried out. For measurement of nitrogenase activity, strain S27^T and several reference strains were incubated on solid RCV mineral medium containing 2% glucose (Weaver *et al.*, 1975). After 48 h of incubation at 30 °C, strains were incubated under 2% (v/v) acetylene in air for 2 h, and were then analysed for ethylene production by HP6890 gas chromatography (Berge *et al.*, 2002). As shown in Table 1, strain S27^T exhibited nitrogenase activity.

Whole-cell DNA for PCR amplification was extracted according to standard methods (Sambrook *et al.*, 1989). A 325 bp fragment of the *nifH* gene was amplified by using two degenerate primers for the nitrogenase Fe protein gene and then sequenced as described by Ding *et al.* (2005). Phylogenetic trees were inferred using the neighbour-joining method and the maximum-parsimony method in

Table 1. Nitrogenase activity of strain S27^T compared with some nitrogen-fixing species of the genus *Paenibacillus*Results are means $\pm$ SD of three determinations.

Strain	Nitrogenase activity [nmol C ₂ H ₄ (mg protein) ⁻¹ h ⁻¹]
<i>P. durus</i> ATCC 35681	1623 $\pm$ 89.4
<i>P. zanthoxyli</i> DSM 18202 ^T	4326 $\pm$ 127.8
<i>P. sabinae</i> DSM 17841 ^T	287 $\pm$ 73.5
<i>P. forsythiae</i> DSM 17842 ^T	268 $\pm$ 55.4
<i>P. odorifer</i> TOD45 ^T	13.4 $\pm$ 1.33
<i>P. polymyxa</i> ATCC 842 ^T	8.74 $\pm$ 0.84
<i>P. graminis</i> RSA19 ^T	183.5 $\pm$ 33.7
S27 ^T	232.3 $\pm$ 43.1

the software package MEGA3.1 (Kumar & Tamura, 2004). No significant differences were found among the phylogenetic trees reconstructed by these two different methods. Phylogenetic analysis based on *nifH* gene sequence revealed that strain S27^T clustered with species of the genus *Paenibacillus* (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). Levels of *nifH* gene sequence similarity between strain S27^T and *P. forsythiae*, *P. zanthoxyli*, *P. durus* and *P. sabinae* were 96.9 %, 96.3 %, 95.1 % and 79.6 %, respectively.

A nearly complete sequence of the 16S rRNA gene (1463 bp) was obtained from a PCR product amplified from strain S27^T by using the universal forward primer P1 and the universal reverse primer P6. Primer P1 (5'-AGAGTTTGATCCTGGTCAGAACGCT-3') corresponds to positions 8–37 and primer P6 (5'-TACGGCTA-CCTTGTACGACTTCACCCC-3') corresponds to positions 1479–1506 in the *Escherichia coli* 16S rRNA gene (Yanagi & Yamasato, 1993). The amplified 16S rRNA gene was sequenced by using an ABI377 automatic sequencer (Applied Biosystems). Comparison of the 16S rRNA gene sequence of strain S27^T with sequences held in GenBank

revealed that this organism clustered with species of the genus *Paenibacillus*. The 16S rRNA gene sequence of strain S27^T was aligned with sequences of recognized species of the genus *Paenibacillus* by using the software package MEGA3.1 (Fig. 2 and Supplementary Fig. S2). An extended version of this tree is available as Supplementary Fig. S3. Strain S27^T showed high 16S rRNA gene sequence similarities with *P. durus* DSM 1735^T (97.3 %), *P. sabinae* DSM 17841^T (96.9 %), *P. forsythiae* DSM 17842^T (96.7 %) and *P. zanthoxyli* DSM 18202^T (96.6 %). Levels of 16S rRNA gene sequence similarity between strain S27^T and other recognized members of the genus *Paenibacillus* were below 96.5 %.

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 universal reverse primer 1377R (5'-GGCATGCTGATCCGCGATTACTAGC-3') (Shida *et al.*, 1996). A 900 bp fragment was PCR-amplified from strain S27^T when the *Paenibacillus* specific primers PAEN515F and 1377R were used in this study. The result further suggested that strain S27^T was a member of the genus *Paenibacillus*.

Strain S27^T was tested for a range of phenotypic, physiological and biochemical characteristics together with the type strains of closely related species of the genus *Paenibacillus*, including *P. durus* DSM 1735^T, *P. sabinae* DSM 17841^T, *P. forsythiae* DSM 17842^T, *P. zanthoxyli* DSM 18202^T and *P. stellifer* DSM 14472^T. For observation of cell morphology, strain S27^T was incubated on endospore-forming medium [I^{-1} : 0.07 % yeast extract, 0.1 % tryptone, 0.1 % glucose, 0.02 % (NH₄)₂SO₄, 0.02 % MgSO₄·7H₂O and 0.1 % K₂HPO₄, pH 7.0] for 14 h and cells were then examined by scanning electron microscopy (Hitachi S-3400). Strain S27^T formed ellipsoidal spores, located centrally in swollen sporangia (Supplementary Fig. S4). Colonies on LD medium [I^{-1} : 10 g tryptone, 5 g yeast extract, 2.5 g NaCl, pH 6.0] agar were circular, convex,

Fig. 1. Maximum-parsimony phylogenetic tree based on partial *nifH* gene sequence comparison (325 nt fragment), showing the position of strain S27^T. *Mesorhizobium ciceri* UPM-Ca7^T was used as an outgroup. Bootstrap analyses were performed with 1000 replications (percentages are shown at branch points). Bar, 10 substitutions per nucleotide position.

Fig. 2. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences showing the position of strain S27^T among species of the genus *Paenibacillus*. *Bacillus subtilis* NCDO 1769^T was used as an outgroup. Bootstrap analyses were performed with 1000 replications (percentages are shown at branch points). Bar, 20 substitutions per nucleotide position.

white and glossy with entire margins after 72 h of incubation at 30 °C. Gram staining demonstrated that cells of strain S27^T were Gram-stain-positive rods. Cell motility was determined by observing the growth spread of cells in test tubes containing semi-solid LD medium after incubation for 3 days. From the spread morphology of the clone in the test tube, cells of strain S27^T were identified as motile. To determine aerotactic ability, bacterial cells were incubated by mixing with semi-solid medium at 40–50 °C in test tubes followed by incubation at 30 °C for 3 days. Although cells of strain S27^T grew throughout the medium in test tubes, cells near the surface grew better than those near the bottom, suggesting that strain S27^T was facultatively anaerobic.

Most physiological and biochemical tests, including Gram staining, nitrate reduction, production of dextrin, optimal temperature and pH for growth, activities of catalase and oxidase, 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). Hydrolysis of casein and starch was determined as described by Cowan & Steel (1965). Utilization of various substrates as carbon and energy sources was determined as described by Shirling & Gottlieb (1966). The physiological and biochemical characteristics of strain S27^T compared with those of the type strains of closely related species of the genus *Paenibacillus* are presented in Table 2. As shown, strain S27^T has physiological properties that allow its distinction from these recognized species of the genus *Paenibacillus*.

The DNA G + C content was determined by the method of Tamaoka & Komagata (1984). The DNA G + C content of strain S27^T was 46.0 mol% (Table 2), which was within the range of values for recognized species of the genus *Paenibacillus* (Shida *et al.*, 1997).

For DNA–DNA hybridization experiments, total genomic DNA was extracted and purified according to the method described by Yoon *et al.* (1996) with some modifications. DNA–DNA hybridization was performed according to the method of Ziemke *et al.* (1998). DNA–DNA hybridization

values between strain S27^T and *P. durus* DSM 1735^T, *P. sabiniae* DSM 17841^T, *P. forsythiae* DSM 17842^T and *P. zanthoxyli* DSM 18202^T were 37.64 %, 23.12 %, 25.6 % and 34.99 %, respectively. According to the recommendations of Wayne *et al.* (1987), strain S27^T and the four type strains did not belong to the same species as the value for DNA–DNA relatedness was lower than the threshold value of 70 %.

Table 2. Differential phenotypic characteristics between strain S27^T and the type strains of its closest neighbours in the genus *Paenibacillus*

Strains: 1, S27^T; 2, *P. durus* DSM 1735^T; 3, *P. sabiniae* DSM 17841^T; 4, *P. forsythiae* DSM 17842^T; 5, *P. zanthoxyli* DSM 18202^T; 6, *P. stellifer* DSM 14472^T. +, Positive reaction; –, negative reaction.

Characteristic	1	2	3	4	5	6
Optimum growth temp. (°C)	30	30	30	30	30	28
Growth in the presence of 3 % NaCl	–	–	+	+	+	–
Voges–Proskauer reaction	–	+	+	+	+	–
Nitrate reduction	+	–	+	+	+	–
Production of dextrin	–	+	–	–	–	+
Hydrolysis of:						
Casein	–	–	–	+	–	–
Starch	–	+	–	–	–	+
Acid production from:						
Maltose	+	+	–	–	–	+
Sucrose	–	+	–	–	–	+
D-Xylose	+	+	–	–	–	+
Sodium citrate	+	–	–	–	–	–
Sodium succinate	–	–	–	+	–	–
D-Fructose	–	–	–	–	–	+
D-Glucose	+	+	–	–	–	+
Glycerol	–	+	–	–	–	–
D-Sorbitol	+	+	–	–	–	–
Lactose	+	+	–	–	–	+
DNA G + C content (mol%)	46.0	50.3	51.8	50.1	54.3	55.6*

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

Cellular menaquinones were extracted and purified as described by Collins (1985) and were then analysed by HPLC. The menaquinone profile (MK-7 as the major component) was in agreement with the description of the genus *Paenibacillus*. Polar lipids extracted by the method of Minnikin *et al.* (1979) were identified by two-dimensional TLC and spraying with specific reagents as described by Collins *et al.* (1980). The major polar lipids presented in strain S27^T were diphosphatidylglycerol, phosphatidylglycerol and lyso-phosphatidylglycerol. Traces of phosphatidylinositol, phosphatidylinositol-methyl and phosphatidylinositol dimannoside were also detected (Supplementary Fig. S5). *meso*-Diaminopimelic acid as the diagnostic diamino acid in the cell wall was identified by TLC (Staneck & Roberts, 1974). For determination of its cellular fatty acid composition, strain S27^T was incubated in LD medium at 30 °C for 3 days. Analysis was carried out as described by Komagata & Suzuki (1987) by using the Sherlock Identification System (MIDI; Sasser *et al.*, 2005). Profiles of the major fatty acids of strain S27^T and the type strains of closely related members of the genus *Paenibacillus* are shown in Table 3. Anteiso-C_{15:0}, the major fatty acid in recognized members of the genus *Paenibacillus*, was also the major fatty acid component of strain S27^T (36.03 %).

In summary, phylogenetic analysis based on the full-length 16S rRNA gene sequence and the fragment of the *nifH* gene sequence, DNA G+C content and chemotaxonomic properties revealed that strain S27^T was a member of the genus *Paenibacillus*. Several phenotypic characteristics and DNA–DNA hybridization further demonstrated that the isolated strain, S27^T, represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus sophorae* sp. nov. is proposed.

Description of *Paenibacillus sophorae* sp. nov.

Paenibacillus sophorae (so.pho'ra.e. N.L. gen. n. *sophorae* of *Sophora*, referring to the plant *Sophora japonica*, the source of the rhizosphere soil from which the type strain was isolated).

Cell are Gram-stain-positive, facultatively anaerobic, motile, straight rods, measuring 0.6–1.5 µm × 1.5–3 µm. Ellipsoidal spores are located centrally in swollen sporangia. Colonies on LD medium are circular, convex, white and glossy with entire margins after 72 h of incubation at 30 °C; usually they are 1–2 mm in diameter. The temperature range for growth is 15–50 °C, with optimal growth at 30 °C. The pH range for growth is 4–8 (optimum pH 6.0). Unable to tolerate 3 % (w/v) NaCl. Growth is inhibited by 0.001 % (w/v) lysozyme. Catalase- and oxidase-negative. Negative for the Voges–Proskauer reaction. Nitrate can be reduced to nitrite. Nitrogen is fixed. The following substrates are utilized for growth and acid production: maltose, D-xylose, sodium citrate, D-glucose, lactose, D-sorbitol, raffinose, N-acetyl-D-glucosamine, glycogen, mannitol, trehalose, cellobiose, D-galactose, L-sorbose and D-mannose. Acid is not produced

Table 3. Cellular fatty acid profiles of strain S27^T and type strains of closely related species of the genus *Paenibacillus*

Strains: 1, S27^T; 2, *P. durus* DSM 1735^T; 3, *P. zanthoxyli* DSM 18202^T; 4, *P. forsythiae* DSM 17842^T; 5, *P. sabinae* DSM 17841^T. Data 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*. Results were determined under the same conditions for all strains.

Fatty acid	1	2	3	4	5
Saturated straight-chain					
C _{14:0}	5.37	4.76	4.84	4.79	2.93
C _{15:0}	<0.2	1.33	2.15	1.94	1.55
C _{16:0}	26.85	19.87	12.88	18.59	18.07
C _{18:0}	1.58	5.48	6.77	5.73	3.24
Saturated iso-branched					
iso-C _{14:0}	3.40	5.31	3.79	2.66	3.24
iso-C _{15:0}	4.20	7.98	1.63	0.99	1.40
iso-C _{16:0}	7.98	10.58	14.90	11.53	14.44
iso-C _{17:0}	1.12	1.48	0.59	0.95	0.82
Saturated anteiso-branched					
anteiso-C _{15:0}	36.03	39.76	32.25	29.97	36.62
anteiso-C _{17:0}	3.24	1.94	3.87	4.61	6.34
Unsaturated					
C _{18:1} ω9 <i>c</i>	0.96	2.12	1.54	4.83	3.85

from sucrose, sodium succinate, D-fructose, glycerol, L-aspartate, L-arabinose, sodium DL-malate, D-ribose, inositol, DL-malic acid, inulin or creatine. Cannot hydrolyse casein, starch or gelatin. The DNA G+C content of the type strain is 46.0 mol% (thermal denaturation method). The cell wall contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acids are anteiso-C_{15:0}, C_{16:0} and iso-C_{16:0}. Sensitive to ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹), streptomycin (40 µg ml⁻¹), tetracycline (12.5 µg ml⁻¹) and chloramphenicol (30 µg ml⁻¹) but resistant to spectinomycin (100 µg ml⁻¹).

The type strain, S27^T (=CGMCC 1.10238^T =DSM 23020^T), was isolated from rhizosphere soil of *Sophora japonica* planted in Beijing, China.

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