Paenibacillus sonchi sp. nov., a nitrogen-fixing species isolated from the rhizosphere of *Sonchus oleraceus*

Yuan-Yuan Hong,¹ Yu-Chao Ma,¹ Yu-Guang Zhou,² Fei Gao,³
Hong-Can Liu² and San-Feng Chen¹

Correspondence
San-feng Chen
chensf@cau.edu.cn

¹The State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing 100094, PR China

²China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, PR China

³College of Biological Sciences, China Agricultural University, Beijing 100094, PR China

A nitrogen-fixing bacterium, designated strain X19-5^T, was isolated from rhizosphere soil of *Sonchus oleraceus*. Phylogenetic analysis based on a fragment of the *nifH* gene and the full-length 16S rRNA gene sequence revealed that strain X19-5^T was a member of the genus *Paenibacillus*. Strain X19-5^T showed the highest 16S rRNA gene sequence similarity (98.8 %) with *Paenibacillus graminis* RSA19^T and below 97 % similarity with other recognized members of the genus. The level of DNA–DNA relatedness between strain X19-5^T and *P. graminis* RSA19^T was 45.7 %. The DNA G + C content of strain X19-5^T was 46.8 mol%. The major fatty acids were anteiso-C_{15:0}, C_{16:0} and iso-C_{16:0}. On the basis of its phenotypic characteristics and the level of DNA–DNA hybridization, strain X19-5^T is considered to represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus sonchi* sp. nov. is proposed. The type strain is X19-5^T (=CCBAU 83901^T=LMG 24727^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 physiologically diverse characteristics. Some species consistently show a great capacity to fix atmospheric nitrogen *in vitro* (Berge *et al.*, 2002; Ma *et al.*, 2007a, b; Ding *et al.*, 2005; Elo *et al.*, 2001). Here we show that a bacterial strain, designated X19-5^T, isolated from rhizosphere soil of *Sonchus oleraceus* represents a novel nitrogen-fixing species of the genus *Paenibacillus*.

Strain X19-5^T was isolated from rhizosphere soil of *Sonchus oleraceus* by screening on nitrogen-free medium (Ma *et al.*, 2007a). The nitrogen-free medium comprised (per litre water): 20 g sucrose, 0.1 g K₂HPO₄, 0.4 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 0.01 g FeCl₃ and 0.002 g Na₂MoO₄. One gram of soil was placed in 9 ml sterile water and stirred for 50 min. Aqueous volumes (100 µl of the mixture) were heated at 80 °C for 10 min and then

spread on nitrogen-free medium in triplicate and incubated at 30 °C. After 3 days incubation, strain X19-5^T was selected for further study.

To confirm the nitrogen-fixing capacity of strain X19-5^T, an assay for nitrogenase activity and PCR amplification of the *nifH* gene were carried out. For measurement of nitrogenase activity, strain X19-5^T and several reference strains were grown on solid RCV mineral medium containing 0.5 % glucose, malate, starch and mannitol (Weaver *et al.*, 1975). After 48 h at 30 °C, strains were incubated under 2 % (v/v) acetylene in air for 2 h and then analysed for ethylene production by using gas chromatography (Berge *et al.*, 2002). As shown in Table 1, strain X19-5^T exhibited nitrogenase activity.

Whole-cell DNA for PCR amplification was extracted according to standard methods (Sambrook *et al.*, 1989). A 324 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 and maximum-parsimony methods in the software package MEGA3.1 (Kumar *et al.*, 2004). As no significant differences were found among the phylogenetic trees obtained by the different methods used, only the trees constructed by using the neighbour-joining method are

The GenBank/EMBL/DDBJ accession numbers for the *nifH* and 16S rRNA gene sequences of strain X19-5^T are EU867444 and DQ358736, respectively.

An extended neighbour-joining phylogenetic tree of representatives of the genus *Paenibacillus* based on 16S rRNA gene sequences is available as supplementary material with the online version of this paper.

Table 1. Nitrogenase activity of strain X19-5^T (*P. sonchi* sp. nov.) 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 ₂ (mg protein) ⁻¹]
<i>P. azotofixans</i> ATCC 35681 ^T	2256.8 $\pm$ 46.9
<i>P. polymyxa</i> ATCC 842 ^T	3.2 $\pm$ 0.55
<i>P. macerans</i> ATCC 8244 ^T	2.48 $\pm$ 0.2
<i>P. odorifer</i> TOD 45 ^T	37.62 $\pm$ 18.9
<i>P. graminis</i> RSA19 ^T	272.9 $\pm$ 7.9
<i>P. sabinae</i> DSM 17841 ^T	3885.9 $\pm$ 72.9
<i>P. zanthoxyli</i> DSM 18202 ^T	6282.4 $\pm$ 107.97
Strain X19-5 ^T	129.8 $\pm$ 82.2

shown. Phylogenetic analysis based on *nifH* gene sequences revealed that strain X19-5^T clustered with species of the genus *Paenibacillus* (Fig. 1). Levels of *nifH* gene sequence similarity between strain X19-5^T and *Paenibacillus zanthoxyli* DSM 18202^T, *Paenibacillus azotofixans* ATCC 35681^T, *Paenibacillus forsythiae* DSM 17842^T, *Paenibacillus graminis* RSA19^T, *Paenibacillus macerans* ATCC 8244^T, *Paenibacillus odorifer* TOD45^T, *Paenibacillus polymyxa* DSM 36^T and *Paenibacillus sabinae* DSM 17841^T were 97.6, 97.6, 97.5, 98.1, 98.1, 98.4, 98.1 and 97.5 %, respectively.

A nearly complete sequence of the 16S rRNA gene (1500 bp) was obtained from a PCR product amplified from strain X19-5^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'-TACGGCTACCTTGTTACGACTTCACCCC-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 X19-5^T with sequences held in GenBank revealed that this organism clustered with species of the genus *Paenibacillus*. The 16S rRNA gene sequence of strain X19-5^T was aligned with sequences of recognized species of the genus *Paenibacillus* by using the software package MEGA3.1. (Fig. 2; an extended version of this tree is available as Supplementary Fig. S1, in IJSEM Online). Strain X19-5^T showed the highest 16S rRNA gene sequence similarity with *P. graminis* RSA19^T (98.8 %). Levels of 16S rRNA gene sequence similarity between strain X19-5^T and other recognized members of the genus *Paenibacillus* were below 97 %.

Strain X19-5^T was tested for a range of phenotypic, physiological and biochemical characteristics together with the type strains of closely related *Paenibacillus* species, including *P. azotofixans* ATCC 35681^T, *P. odorifer* TOD45^T,

Fig. 1. Phylogenetic tree based on partial *nifH* gene sequence comparison (324 nt fragment) obtained using the neighbour-joining method, showing the position of strain X19-5^T among species of the genus *Paenibacillus*. The sequence of *Mesorhizobium ciceri* was used as an outgroup. Numbers at nodes are percentage bootstrap values based on 1000 resamplings. Bar, 0.2 substitutions per nucleotide position.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain X19-5^T among species of the genus *Paenibacillus*. The sequence of *Bacillus subtilis* NCDO 1769^T was used as an outgroup. Numbers at nodes are percentage bootstrap values based on 1000 resamplings. Bar, 0.01 substitutions per nucleotide position. An extended version of the tree including all recognized species of *Paenibacillus* is available as Supplementary Fig. S1 (in IJSEM Online).

P. forsythiae DSM 17842^T, *P. zanthoxyli* DSM 18202^T, *P. sabinae* DSM 17841^T, *P. graminis* RSA19^T and *P. stellifer* DSM 14472^T, as reference strains. For observation of cell morphology, strain X19-5^T was grown in endospore-forming medium [0.07% yeast extract, 0.1% tryptone, 0.1% glucose, 0.02% (NH₄)₂SO₄, 0.02% MgSO₄·7H₂O and 0.1% K₂HPO₄, pH 7.2] for 72 h, and the cells were then examined by light microscopy. Strain X19-5^T formed ellipsoidal spores, located centrally or subterminally in swollen sporangia. Colonies on LD medium [per litre water: 10 g tryptone, 5 g yeast extract, 2.5 g NaCl, pH 7.0] agar were circular, convex and glossy with entire margins after 72 h incubation. Gram-staining demonstrated that cells of strain X19-5^T were Gram-positive rods. Cell motility was determined by using two methods, i.e. directly by light microscopy and observation of growth spread of cells in test tubes containing semisolid LD medium after 3 days incubation. Cells of strain X19-5^T were motile. To determine aerotactic ability, bacterial cells were inoculated by mixing them with semisolid medium at 40–50 °C in test tubes followed by growth at 30 °C for 3 days. Although cells of strain X19-5^T grew throughout the medium in test tubes, cells near the surface grew better than those near the bottom, suggesting that strain X19-5^T was aerobic.

Most physiological and biochemical tests, including Gram staining, nitrate reduction, production of dextrin, temperature and pH optima for growth, activities of catalase and

oxidase and 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). 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). Differential physiological and biochemical characteristics of strain X19-5^T and those of the type strains of closely related *Paenibacillus* species are given in Table 2. Strain X19-5^T had physiological properties that allowed its distinction from recognized *Paenibacillus* species.

The DNA G+C content was determined by using the method of Tamaoka & Komagata (1984). The DNA G+C content of strain X19-5^T was 46.8 mol% (Table 2), within the range of values for recognized *Paenibacillus* species (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). The level of DNA–DNA relatedness between strain X19-5^T and *P. graminis* RSA19^T was 45.7%. According to the recommendations of the ad hoc committee, strain X19-5^T and *P. graminis* RSA19^T did not belong to the same species as the value found for

Table 2. Differential phenotypic characteristics between strain X19-5^T and the type strains of selected *Paenibacillus* species

Strains: 1, X19-5^T (*P. sonchi* sp. nov.); 2, *P. azotofixans* ATCC 35681^T (data from Ma *et al.*, 2008); 3, *P. odorifer* TOD45^T (von der Weid *et al.*, 2002); 4, *P. graminis* RSA19^T (von der Weid *et al.*, 2002); 5, *P. stellifer* DSM 14472^T (Suominen *et al.*, 2003); 6, *P. forsythiae* DSM 17842^T (Ma & Chen, 2008); 7, *P. sabinae* DSM 17841^T (Ma *et al.*, 2007a); 8, *P. zanthoxyli* DSM 18202^T (Ma *et al.*, 2007b). +, Positive reaction; –, negative reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8
Optimum growth temperature (°C)	30	30	30	30	28	30	30	30
Growth with lysozyme (0.001 %)	+	–	+	–	ND	–	–	–
Voges–Proskauer	+	+	–	–	ND	+	+	+
Nitrate reduction	–	+	+	+	–	+	+	+
Production of dextrin	+	–	–	–	+	–	–	–
Hydrolysis of:								
Casein	+	–	–	–	ND	+	–	–
Starch	+	–	–	–	+	ND	–	–
Acid produced from:								
Sucrose	–	–	+	–	+	–	–	–
Glucose	–	+	+	+	+	–	–	–
Maltose	–	+	+	+	+	–	–	–
Lactose	–	–	ND	ND	+	–	–	–
Sodium succinate	–	–	–	–	–	+	–	–
D-Xylose	–	–	–	–	+	ND	–	–
Fructose	+	–	–	–	+	ND	–	–
L-Aspartate	–	+	+	+	–	–	–	–
DNA G + C content (mol%)	46.83	50.7	44	52.1	55.6	50.4	51.9–52.9	52.3–54.4

DNA–DNA relatedness was lower than the threshold value of 70 % (Wayne *et al.*, 1987).

For the determination of its cellular fatty acid composition, strain X19-5^T was grown 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 X19-5^T and the type strains of closely related *Paenibacillus* species are shown in Table 3. Anteiso-

Table 3. Cellular fatty acid profiles of strain X19-5^T and closely related *Paenibacillus* type strains

Strains: 1, X19-5^T (*P. sonchi* sp. nov.); 2, *P. graminis* RSA19^T (data from von der Weid *et al.*, 2002); 3, *P. borealis* DSM 13188^T (data from Elo *et al.*, 2001); 4, *P. stellifer* DSM 14472^T (Suominen *et al.*, 2003); 5, *P. azotofixans* ATCC 35681^T (Yoon *et al.*, 2003); 6, *P. zanthoxyli* DSM 18202^T (Ma *et al.*, 2007b); 7, *P. forsythiae* DSM 17842^T (Ma & Chen, 2008); 8, *P. sabinae* DSM 17841^T (Ma *et al.*, 2007a). Data are percentages of the total fatty acids. NR, Not reported. 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*.

Fatty acid	1	2	3	4	5	6	7	8
Saturated straight-chain								
C _{14:0}	4.31	3.69	18.6	0.7	5.0	4.87	4.85	2.96
C _{16:0}	19.09	14.98	10.0	9.1	15.5	12.83	18.7	18.19
C _{18:0}	1.28	2.36	NR	1.1	0.3	6.74	5.64	3.28
Saturated iso-branched								
iso-C _{14:0}	9.80	12.20	4.9	0.6	4.7	3.99	2.64	3.20
iso-C _{15:0}	3.35	4.22	11.5	5.5	8.7	1.51	0.97	1.37
iso-C _{16:0}	15.85	13.42	10.5	7.7	5.3	14.85	11.48	14.40
iso-C _{17:0}	0.64	0.53	4.90	7.0	1.1	0.61	0.98	0.79
Saturated anteiso-branched								
anteiso-C _{15:0}	39.36	42.00	35.2	49.9	45.4	32.19	29.87	36.59
anteiso-C _{17:0}	2.01	1.45	2.1	16.7	2.1	3.89	4.52	6.38
Unsaturated								
C _{18:1} ω 9 <i>c</i>	0.39	0.77	0.4	<0.2	<0.2	1.51	4.87	3.88

C_{15:0}, the major fatty acid in recognized members of the genus *Paenibacillus*, was also the major fatty acid component of strain X19-5^T (39.3 %).

In summary, phylogenetic analysis based on the full-length 16S rRNA gene sequence and a fragment of the *nifH* gene sequence, DNA G+C content and chemotaxonomic properties revealed that strain X19-5^T was a member of the genus *Paenibacillus*. Several phenotypic characteristics and levels of DNA–DNA hybridization further demonstrated that our isolate represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus sonchi* sp. nov. is proposed, strain X19-5^T being the type strain.

Description of *Paenibacillus sonchi* sp. nov.

Paenibacillus sonchi (son'chi. L. n. *sonchus* -i, the herb sow-thistle, and also a botanical genus name; L. gen. n. *sonchi* of *Sonchus*, referring to the plant *Sonchus oleraceus*, the source of the rhizosphere soil from which the type strain was isolated).

Gram-positive, aerobic, motile, straight rods (0.5 × 5 µm). Ellipsoidal spores are located centrally or subterminally in swollen sporangia. Colonies on LD medium are circular, convex and glossy with entire margins after incubation for 72 h at 30 °C. Temperature range for growth is 10–40 °C, with optimum growth at 30 °C. Optimum pH is 7.0–7.2. Grows in the absence of NaCl and in 3 % (w/v) NaCl, but is unable to tolerate 5 % NaCl. Growth is not inhibited by 0.001 % (w/v) lysozyme. Catalase-negative and oxidase-negative. Positive for the Voges–Proskauer reaction. Nitrate is not reduced to nitrite. Nitrogen is fixed. Able to utilize fructose to produce acid, but not glucose, sucrose, lactose, succinate, D-xylose, maltose or L-aspartate. Casein and starch are hydrolysed. The G+C content of the DNA of the type strain is 46.8 mol% (thermal denaturation method).

The type strain, X19-5^T (=CCBAU 83901^T=LMG 24727^T), was isolated from rhizosphere soil of *Sonchus oleraceus* in Xinjiang, China.

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