Paenibacillus zanthoxyli sp. nov., a novel nitrogen-fixing species isolated from the rhizosphere of *Zanthoxylum simulans*

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Five endospore-forming, nitrogen-fixing strains were isolated from rhizosphere soils of *Zanthoxylum simulans* planted in Beijing, China. Phylogenetic analysis based on full-length 16S rRNA gene sequences revealed that the five strains formed a distinct cluster within the genus *Paenibacillus*. High levels of 16S rRNA gene sequence similarity were found between these novel strains and *Paenibacillus azotofixans* ATCC 35681^T (97.8–98.5 % similarity) and *Paenibacillus stellifer* DSM 14472^T (95.4–96.3 %). Levels of 16S rRNA gene sequence similarity between the novel isolates and other species of the genus *Paenibacillus* were less than 95.0 %. Levels of 16S rRNA gene sequence similarity among the isolates were more than 98.0 %. DNA–DNA relatedness between the five novel isolates and *P. azotofixans* ATCC 35681^T was 45.50–47.45 % and relatedness among the five novel strains was 95.8–99.6 %. A significant feature of the novel strains that differentiated them from *P. azotofixans* and other *Paenibacillus* species was that none of the novel strains could produce acid or gas from the following various carbohydrates: glucose, sucrose, lactose, fructose, glycerol, xylose, maltose, D-sorbitol, sodium succinate, sodium citrate, glycine or L-aspartate. Anteiso-branched C_{15:0} was the major fatty acid component (36.59 %) of novel strain JH29^T. On the basis of phenotypic properties, 16S rRNA gene sequences, DNA G + C content, DNA–DNA hybridization, chemotaxonomic properties and the *nifH* gene sequence, the five novel strains form a very homogeneous group which is different from other related species within the genus *Paenibacillus*. Therefore, the five novel strains are considered to represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus zanthoxyli* sp. nov. is proposed. The type strain is JH29^T (= CCBAU 10243^T = DSM 18202^T).

Ash *et al.* (1993) created the genus *Paenibacillus* from the genus *Bacillus* based on their 16S rRNA gene sequences. Members of the genus *Paenibacillus* are aerobic or facultatively anaerobic, rod-shaped, endospore-forming bacteria that are widely distributed in nature (Claus & Berkeley, 1986; Slepecky & Hemphill, 1991). Nitrogen fixation has been described in some species of *Paenibacillus*, such as *Paenibacillus polymyxa* (Grau & Wilson, 1962), *Paenibacillus*

macerans (Witz *et al.*, 1967), *Paenibacillus azotofixans* (Seldin *et al.*, 1984), *Paenibacillus peoriae* (Elo *et al.*, 2001), *Paenibacillus graminis*, *Paenibacillus odorifer* (Berge *et al.*, 2002) and *Paenibacillus brasiliensis* (von der Weid *et al.*, 2002). It has been reported that *P. azotofixans* has the highest nitrogenase activity among these nitrogen-fixers (Achouak *et al.*, 1999; Berge *et al.*, 2002). *P. azotofixans* has been found in Brazilian and Hawaiian soils, but not in soils from temperate zones, while *P. polymyxa* has been isolated from various environments around the world on different plant roots (Berge *et al.*, 2002).

Here we report the isolation of five novel strains from the rhizosphere of *Zanthoxylum simulans* (prickly-ash). The five novel strains have nitrogenase activities higher than that of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Paenibacillus zanthoxyli* sp. nov. JH29^T is DQ471303.

A more detailed phylogenetic tree that includes additional recognized species of the genus *Paenibacillus* is available as a supplementary figure in IJSEM Online.

P. azotofixans and their 16S rRNA gene sequences have a high level of similarity to that of *P. azotofixans*. The strains were identified as a novel species of the genus *Paenibacillus* based on phenotypic properties, 16S rRNA gene sequences, DNA G+C content, DNA–DNA hybridization, chemotaxonomic properties and *nifH* gene sequence.

The bacterial strains examined were isolated from the rhizosphere soil of prickly-ash planted in Beijing, China. A 1 g sample of soil was placed in 9 ml sterile water and stirred for 50 min. Aqueous portions (100 µl) were heated at 80 °C for 10 min and then spread on nitrogen-free medium in triplicate and incubated at 30 °C. The nitrogen-free medium used for the isolation of bacterial strains contained (l⁻¹): 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. After 5 days incubation, five strains, designated JH8, JH29^T, JH31, JT34 and JH95 were selected for further study.

To determine cell morphology, the five novel strains were 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 7.2] for 72 h. Cell morphology was examined by light microscopy. Catalase activity was analysed by bubble formation in a 3 % (w/v) H₂O₂ solution. Hydrolysis of casein, gelatin 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). Tolerance to NaCl was measured in a medium [0.5 % peptone, 0.3 % (w/v) beef extract] containing NaCl at concentrations of 3 and 5 % (w/v). Other physiological and biochemical tests were also performed using conventional methods (Priest *et al.*, 1981; Rhodes-Roberts, 1981).

Analysis of cellular fatty acid contents was carried out as described by Komagata & Suzuki (1987) using the Sherlock Identification System (MIDI).

To determine the nitrogen-fixing capability of the five strains, an assay for nitrogenase activity and PCR amplification of the *nifH* gene were carried out. A 324 bp fragment of the *nifH* gene was isolated from the five novel strains as described by Ding *et al.* (2005). Nitrogenase activity was determined by the acetylene-reduction assay method (Berge *et al.*, 2002).

Chromosomal DNA was extracted and purified according to standard method (Sambrook *et al.*, 1989). The 16S rRNA gene sequence was amplified as described by Yanagi & Yamasato (1993) and sequenced (Ding *et al.*, 2005). Sequences were aligned using CLUSTAL_X software (Thompson *et al.*, 1997). Evolutionary distances were calculated using the DNADIST program from the TREECONW software package. A phylogenetic tree was generated by the neighbour-joining method using the TREECONW software package (Van der Peer & De Wachter, 1994).

DNA–DNA hybridization and DNA G+C content were determined as described by De Ley *et al.* (1970).

The nearly complete 16S rRNA gene sequences of the five novel strains were compared with sequences held in GenBank. This analysis indicated that the novel strains are phylogenetically related to members of the genus *Paenibacillus*. A neighbour-joining phylogenetic tree showing the position of the novel strains in relation to some other *Paenibacillus* species is shown in Fig. 1. A more detailed phylogenetic tree that includes additional recognized species of the genus *Paenibacillus* is available as Supplementary Fig. S1 in IJSEM Online. The five novel strains showed high levels of 16S rRNA gene sequence similarity with *P. azotofixans* ATCC 35681^T (97.81–98.5 %) and *Paenibacillus stellifer* DSM 14472^T (95.39–96.25 %). Levels of 16S rRNA gene sequence similarity between the five novel strains and other species of *Paenibacillus* were less than 95.0 %. Levels of 16S rRNA gene sequence similarity among the five novel strains were more than 98.0 %.

Fig. 1. Phylogenetic tree based on neighbour-joining analysis of the 16S rRNA gene sequence of *P. zanthoxyli* sp. nov. compared with other *Paenibacillus* species. *Escherichia coli* was used as an outgroup. Bootstrap analyses were performed with 1000 cycles. Only bootstrap values > 50 % are shown at the branch points. Bar, 0.1 substitutions per nucleotide position.

Table 1. Phenotypic characteristics of *P. zanthoxyli* sp. nov. and other nitrogen-fixing *Paenibacillus* species

Taxa: 1, *P. zanthoxyli* sp. nov. (five strains tested); 2, *P. azotofixans* ATCC 35681^T; 3, *P. polymyxa* ATCC 842^T; 4, *P. macerans* ATCC 8244^T; 5, *P. odorifer* TOD45^T; 6, *P. graminis* RSA19^T; 7, *P. stellifer* DSM 14472^T; 8, *P. brasilensis* DSM 14914^T; 9, *P. peoriae* DSM 8320^T. Data for taxa 5, 6, 8 and 9 are from von der Weid *et al.* (2002). Data for taxon 7 are from Suominen *et al.* (2003). +, Positive; –, negative; v, variable; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9
Optimum growth temp. (°C)	30	30	30	30	30	30	28	30	30
Growth with lysozyme (0.001 %)	–	–	–	+	+	–	ND	+	+
Voges–Proskauer test	+	+	+	+	–	–	ND	+	+
Nitrate reduction	+	+	+	+	+	+	–	+	+
Production of dextrin	–	–	–	+	–	–	+	–	–
Hydrolysis of:									
Casein	–	–	–	–	–	–	ND	+	+
Gelatin	–	–	–	–	–	–	ND	v	+
Starch	–	–	–	–	–	–	+	+	+
Acid produced from:									
L-Aspartate	–	+	+	+	+	+	–	ND	ND
Fructose	–	–	–	–	–	–	+	+	–
Glucose	–	+	+	+	+	+	+	+	ND
Glycerol	–	–	+	+	–	–	–	–	v
Lactose	–	–	+	+	ND	ND	+	+	+
Maltose	–	+	+	+	+	+	+	ND	ND
Sodium citrate	–	–	–	–	–	–	–	+	+
Sodium succinate	–	–	–	+	–	–	–	+	+
D-Sorbitol	–	–	+	+	–	–	–	–	+
Sucrose	–	–	+	+	+	–	+	ND	+
D-Xylose	–	–	–	–	–	–	+	–	+
DNA G + C (mol%)	52.3–54.4	50.7	43–46	49–51	44	52.1	55.6	ND	45–47

The five novel strains are facultatively anaerobic, Gram-positive, motile, straight rods (0.35–0.4 × 4.0–4.8 µm). They produce ellipsoidal or oval spores that are located subterminally or centrally in swollen sporangia. Colonies of the strains on nutrient agar are circular, convex and glossy with entire margins. The phenotypic characteristics of the novel strains and reference *Paenibacillus* species are presented in

Table 1. The five novel strains were found to have physiological properties that enabled them to be distinguished from these recognized *Paenibacillus* species. The DNA G + C contents of strains JH29, JH8, JH31, JH34 and JH95 were 52.3–54.4 mol% (Table 1), which are in the range characteristic of recognized *Paenibacillus* species (Shida *et al.*, 1997).

Fig. 2. Phylogenetic tree based on partial *nifH* gene sequences, showing the position of the five novel strains in relation to other bacterial species from the GenBank database. Bootstrap analyses were performed with 1000 cycles. Only bootstrap values > 50 % are shown at the branch points. Bar, 0.1 substitutions per nucleotide position.

PCR amplification demonstrated that all five novel strains possess the *nifH* gene. Phylogenetic analysis based on *nifH* sequences revealed that the five strains clustered together with species of the genus *Paenibacillus* (Fig. 2). The five novel strains showed high levels of *nifH* gene sequence similarity with *P. azotofixans* ATCC 35681^T (97.20–98.15 %), *P. polymyxa* (93.18–95.45 %) and *P. odorifer* (88.79 %). Levels of *nifH* gene sequence similarity among the five novel strains were 97.22–100 %.

The nitrogenase activity of the five novel isolates and *P. azotofixans*, *P. polymyxa*, *P. macerans*, *P. odorifer* and *P. graminis* was also tested by the acetylene-reduction assay method (Berge *et al.*, 2002). As shown in Table 2, all of the novel strains exhibited significant nitrogenase activity.

DNA–DNA hybridization tests were performed to determine the genomic relatedness between the five novel strains and the type strain of *P. azotofixans*. DNA–DNA relatedness between the novel strains and *P. azotofixans* ATCC 35681^T was 45.50–47.45 % and among the five novel strains the relatedness value was 95.8–99.6 %.

The cellular fatty acid profile of one of the novel isolates, JH29^T, was determined together with those of *P. azotofixans* ATCC 35681^T, *P. stellifer* DSM 4472^T, *P. polymyxa* DSM 36^T and *P. macerans* ATCC 8244^T (Table 3). Anteiso-branched C_{15:0}, the major fatty acid characteristic for the genus *Paenibacillus*, was also the major fatty acid component (32.19 %) of strain JH29^T.

In conclusion, on the basis of phenotypic properties, 16S rRNA gene sequences, DNA G+C content, DNA–DNA hybridization, chemotaxonomic properties and *nifH* gene sequence, the five novel strains form a very homogeneous group which is different from other related species within the genus *Paenibacillus*. Therefore, we propose that they represent a novel species of the genus *Paenibacillus*, for

Table 2. Nitrogenase activity of the five novel strains and nitrogen-fixing *Paenibacillus* reference strains

Values are $\pm$ standard deviation.

Strain	Activity [nmol C ₂ H ₄ (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> TOD45 ^T	37.62 $\pm$ 18.9
<i>P. graminis</i> RSA19 ^T	272.9 $\pm$ 7.9
<i>P. zanthoxyli</i> sp. nov.	
JH29 ^T	6282.4 $\pm$ 107.97
JH8	2815.7 $\pm$ 211.4
JH31	2702.2 $\pm$ 81.2
JT34	2526.6 $\pm$ 24.2
JH95	1872.1 $\pm$ 184.3

Table 3. Cellular fatty acid profiles (%) of *P. zanthoxyli* sp. nov. JH29^T and closely related *Paenibacillus* species

Strains: 1, *P. zanthoxyli* sp. nov. JH29^T; 2, *P. azotofixans* ATCC 35681^T (Yoon *et al.*, 2003); 3, *P. stellifer* DSM 14472^T (Suominen *et al.*, 2003); 4, *P. polymyxa* DSM 36^T (Yoon *et al.*, 2003); 5, *P. macerans* ATCC 8244^T (Elo *et al.*, 2001). NR, Not reported. Only selected fatty acids of strain JH29^T are presented for comparison.

Fatty acid	1	2	3	4	5
Saturated straight-chain					
C _{14:0}	4.87	5.0	0.7	5.0	3.7
C _{15:0}	2.07	2.2	0.5	3.4	0.5
C _{16:0}	12.83	15.5	9.1	20.5	17.9
C _{18:0}	6.74	0.3	1.1	0.3	NR
Saturated iso-branched					
C _{14:0}	3.99	4.7	0.6	4.7	1.8
C _{15:0}	1.51	8.7	5.5	8.7	2.6
C _{16:0}	14.85	5.3	7.7	NR	17.1
C _{17:0}	0.61	1.1	7.0	NR	4.1
Saturated anteiso-branched					
C _{13:0}	0.49	NR	NR	1.8	<0.2
C _{15:0}	32.19	45.4	49.9	45.5	34.5
C _{17:0}	3.89	2.1	16.7	2.1	16.1
Unsaturated*					
C _{18:1ω5c}	2.02	NR	NR	1.8	NR
C _{18:1ω7c}	1.78	NR	NR	NR	NR
C _{18:1ω9c}	1.51	<0.2	<0.2	<0.2	<0.2

*The position of the double bond can be located by counting from the methyl (ω) end of the carbon chain. A *cis* isomer is indicated by the suffix *c*.

which the name of *Paenibacillus zanthoxyli* sp. nov. is proposed.

Description of *Paenibacillus zanthoxyli* sp. nov.

Paenibacillus zanthoxyli (zan'th.ox.y.li. N.L. gen. n. *zanthoxyli* of *Zanthoxylum*, referring to the plant *Zanthoxylum simulans*, the source of the rhizosphere soil from which the type strain was isolated).

Gram-positive, facultatively anaerobic, motile, straight rods (0.35–0.4 $\times$ 4.0–4.8 μ m). Oval or ellipsoidal endospore located subterminally or centrally in swollen sporangia. Colonies on nutrient agar are circular, convex and glossy with entire margins. Growth occurs at pH 4.2–10.0 and at 4–37 °C. Growth occurs in 3 % (w/v) NaCl, but not in 0.001 % (w/v) lysozyme. Catalase activity is positive and oxidase activity is negative. Voges–Proskauer and methyl-red reactions are positive. Nitrogen fixation is detected by the presence of the *nifH* gene and acetylene reduction in all strains. Acid and gas are not produced from glucose, sucrose, lactose, fructose, glycerol, xylose, maltose, D-sorbitol, sodium succinate, sodium citrate, glycine or L-aspartate. Gelatin, casein and starch are not hydrolysed. The major cellular fatty acids of the type strain, JH29^T, are

anteiso-C_{15:0}, iso-C_{16:0} and C_{16:0}. The DNA G + C content of the type strain is 53.2 mol%.

The type strain, JH29^T (= CCBAU 10243^T = DSM 18202^T), was isolated from the rhizosphere soil of *Zanthoxylum simulans* planted in Beijing, China.

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