Paenibacillus sabinae sp. nov., a nitrogen-fixing species isolated from the rhizosphere soils of shrubs

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Five novel endospore-forming, nitrogen-fixing bacterial strains were isolated from the rhizosphere soils of plants of the species *Sabina squamata*, *Weigela florida* and *Zanthoxylum simulans*. A phylogenetic analysis based on 16S rRNA gene sequences revealed that the five strains formed a distinct cluster within the genus *Paenibacillus*. These novel strains showed the highest levels (96.2–98.2%) of 16S rRNA gene sequence similarity with *Paenibacillus azotofixans*. However, the DNA–DNA relatedness between these novel strains and *P. azotofixans* was 12.9–29.5%. The DNA G+C contents of the five strains were found to be 51.9–52.9 mol%. Phenotypic analyses showed that a significant feature of the novel strains (differentiating them from *P. azotofixans* and other *Paenibacillus* species) is that all of them were unable to produce acid and gas from various carbohydrates such as glucose, sucrose, lactose and fructose. Anteiso-branched C_{15:0} was the major fatty acid present in the novel type strain. On the basis of these data, the five novel strains represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus sabinae* sp. nov. is proposed. The type strain is T27^T (=CCBAU 10202^T = DSM 17841^T).

The genus *Paenibacillus* was first proposed by Ash *et al.* (1993) on the basis of phylogenetic data from 16S rRNA gene sequences. At that time, the genus *Paenibacillus* encompassed 11 species, including the nitrogen-fixing species *Paenibacillus polymyxa* (Grau & Wilson, 1962), *Paenibacillus macerans* (Witz *et al.*, 1967) and *Paenibacillus azotofixans* (Seldin *et al.*, 1983, 1984). Since then, continual transfers of *Bacillus* species to the genus and descriptions of novel *Paenibacillus* species have increased the number of recognized *Paenibacillus* species considerably (Tcherpakov *et al.*, 1999; Van der Maarel *et al.*, 2000; Elo *et al.*, 2001). At the time of writing, in addition to the three species mentioned above, the following *Paenibacillus* species have been described as being nitrogen-fixers (on the basis of showing positive nitrogenase activity with the acetylene-reduction method or a PCR-amplified *nifH* fragment): *P. peoriae* and *P. borealis* (Elo *et al.*, 2001), *P. graminis* and *P. odorifer* (Berge *et al.*, 2002), *P. brasiliensis* (von der Weid *et al.*, 2002), *P. wynnii* (Rodríguez-Díaz *et al.*, 2005) and *P. massiliensis* (Ding *et al.*, 2005). Almost all of the

nitrogen-fixing *Paenibacillus* species described were isolated from the rhizosphere soils or roots of graminaceous plants. Here we describe the isolation and classification of a novel nitrogen-fixing species within the genus *Paenibacillus*.

The bacterial strains studied here were isolated from the rhizosphere soils of three species of shrubs, namely single-seed savin (*Sabina squamata*), brocadebeldflower (*Weigela florida*) and pricklyash (*Zanthoxylum simulans*). Soil samples were collected and each sample (1 g) was placed in 9 ml sterile water and stirred for 50 min. Aqueous portions (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. The nitrogen-free medium used for the isolation of bacterial strains contained 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 dissolved in 1 l water. After 5 days incubation, strains T27^T, T49 and T67 were isolated from the rhizosphere soil of the plant *S. squamata*, strain JD2 was from *W. florida* and strain G18-7 was from *Z. simulans*.

As mentioned above, strains were obtained by screening on nitrogen-free medium. To confirm the nitrogen-fixing capabilities of the five strains, an assay for nitrogenase

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of strains T27^T, T67, T49, JD2 and G18-7 are DQ338444–DQ338448 and DQ349125–DQ349129, respectively.

activity and a PCR amplification of the *nifH* gene were carried out. Two degenerate primers (forward primer 5'-GGCTGCGATCC(CGA)AAGGCCGA(CT)TC(CGA)A-CCCG-3' and reverse primer 5'-CTG(GCA)GCCTTGTT-(CT)TCGCGGAT(CG)GGCATGGC-3') were used to amplify a 324 bp fragment of the *nifH* gene from the five strains, as described by Ding *et al.* (2005). Our results showed that the five strains possess *nifH* genes; their GenBank accession numbers are indicated after the bacterial names in Fig. 1. The deduced amino acid sequences of the *nifH* gene products from the five strains were aligned, using CLUSTAL_X software (Thompson *et al.*, 1997) with NifH sequences held in GenBank. A phylogenetic tree was generated using the neighbour-joining method with the software package TREECONW (Van de Peer & De Wachter, 1994). The phylogenetic analysis based on NifH sequences revealed that the five strains clustered together with *Paenibacillus* species (Fig. 1). The five novel strains were also tested for nitrogenase activity by using the acetylene-reduction assay (Berge *et al.*, 2002). All of the strains were grown in screw-capped tubes with 5 ml liquid RCV mineral medium (Weaver *et al.*, 1975) containing 2 % glucose. After 48 h incubation at 30 °C, the caps were replaced by silicone stoppers and 1.5 % of the inner atmosphere of the tube was replaced by acetylene; the tubes were incubated under this atmosphere for 2 h and then analysed for ethylene production by gas chromatography as described by Berge *et al.* (2002). *P. azotofixans*, *P. polymyxa*, *P. macerans*, *P. odorifer* and *P. graminis* were also tested (as reference strains) for their capacity to fix nitrogen by assaying their nitrogenase activities. As shown in Table 1, all of the novel strains exhibited significant nitrogenase activity. These data suggested that these novel stains were nitrogen-fixers.

Strains belonging to the genus *Paenibacillus* are usually identified by PCR amplification of a 16S rRNA gene fragment with the highly specific forward primer PAEN515F (5'-GCTCGGAGAGTGACGGTACCTGAGA-3') and universal reverse primer 1377R (Shida *et al.*, 1997). An 860 bp

Table 1. Nitrogenase activities of the five novel strains and *Paenibacillus* species

Strain	Activity
	nmol C ₂ H ₂ (mg protein) ⁻¹
<i>P. azotofixans</i> ATCC 35681 ^T	2256.8 ± 46.9
<i>P. polymyxa</i> DSM 36 ^T	3.2 ± 0.55
<i>P. macerans</i> ATCC 8244 ^T	2.48 ± 0.2
<i>P. odorifer</i> TOD45 ^T	37.62 ± 18.9
<i>P. graminis</i> RSA19 ^T	272.9 ± 7.9
T27 ^T	3885.9 ± 72.9
T49	2080.4 ± 7.2
T67	1896.1 ± 106.5
JD2	2202.2 ± 81.2
G18-7	2093.8 ± 224.4

fragment was PCR-amplified from each of the novel strains when the *Paenibacillus*-specific primers PAEN515F and 1377R were used in this study. The results suggested that these novel strains were members of the genus *Paenibacillus*.

An almost-complete 16S rRNA gene sequence was amplified from each of the five novel strains, as described by Yoon *et al.* (2002), and the PCR products were sequenced; the GenBank accession numbers of the five novel strains are indicated after the bacterial names in Fig. 2. The 16S rRNA gene sequences of the five novel strains were aligned with those of their most closely related species in the genus *Paenibacillus* by using the CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic analysis based on 16S rRNA gene sequences revealed that the five strains formed one distinct cluster within the genus *Paenibacillus* (Fig. 2). The highest levels of 16S rRNA gene sequence similarity between the five novel strains T27^T, T67, T49, JD2 and G18-7 and *P. azotofixans* were 97.8, 97.6, 97.2, 98.23 and 96.2 %, respectively. The 16S rRNA gene sequence similarities among the five novel strains were 98.2–99.1 %.

Fig. 1. Phylogenetic tree based on partial NifH sequences, showing the position of the five novel strains in relation to other bacterial species from the database. Numbers at branching points represent bootstrap values from 1000 replicates; only values greater than 50 % are shown. Bar, 0.1 substitutions per nucleotide position.

Fig. 2. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic position of the five novel strains with respect to *Paenibacillus* species. *Escherichia coli* was used as an outgroup. Numbers at branching points represent bootstrap values from 1000 replicates; only values greater than 50% are shown. Bar, 0.1 substitutions per nucleotide position.

To determine the cellular morphology, the five bacterial strains were grown on endospore-forming medium [yeast extract (Oxoid), 0.07%; tryptone (Oxoid), 0.1%; glucose,

0.1%; $(\text{NH}_4)_2\text{SO}_4$, 0.02%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02%; K_2HPO_4 , 0.1%; pH 7.2] for 72 h and then the cells were examined by phase-contrast microscopy. The cells were found to be Gram-positive, aerobic, motile and rod-shaped ($0.7\text{--}0.8 \times 2.7\text{--}3.2 \mu\text{m}$), and they produced ellipsoidal or oval spores located subterminally or centrally in swollen sporangia. The colonies of the strains on nutrient agar were circular, convex and glossy with entire margins.

Physiological and biochemical tests were performed using conventional methods. Catalase activity was analysed by bubble formation in a 3% (v/v) H_2O_2 solution. The hydrolysis of casein, gelatin and starch was determined as described by Cowan & Steel (1965). The utilization of various substrates as carbon and energy sources was determined as described by Shirling & Gottlieb (1966). Tolerance of NaCl was measured in a medium [0.5% peptone (Oxoid), 0.3% beef extract (Oxoid)] containing NaCl at concentrations of 3 and 5% (w/v). Other physiological and biochemical tests were also determined using conventional methods. The phenotypic characteristics of the novel strains and several known nitrogen-fixing *Paenibacillus* species are presented in Table 2. A significant feature that differentiated the isolates from *P. azotofixans* and other *Paenibacillus* species was that none of the novel strains produced acid and gas from the following various carbohydrates: glucose, sucrose, lactose, fructose, glycerol, D-xylose, maltose, D-sorbitol, sodium succinate, sodium citrate, glycine and L-aspartate.

DNA G+C contents were determined using the thermal melting protocol (De Ley *et al.*, 1970): the values for the novel strains varied from 51.9 to 52.9 mol%. These values fell within the range for known *Paenibacillus* species (Shida *et al.*, 1997), suggesting that the novel strains belong to that genus.

The levels of DNA–DNA hybridization were determined using the initial renaturation-rate method (De Ley *et al.*, 1970): all of the novel strains displayed 12.9–29.5% DNA–DNA relatedness to *P. azotofixans*. The level of DNA–DNA relatedness varied from 82.6 to 96.0% among the five novel strains. The results indicate that none of these novel strains is related to any known species of *Paenibacillus* and that they all belong to the same novel species.

Analysis of the cellular fatty acid compositions was carried out as described by Komagata & Suzuki (1987), using the Sherlock Identification System (MIDI). The major fatty acid composition of strain T27^T is shown in Table 3 together with those of closely related *Paenibacillus* species. Anteiso-branched $\text{C}_{15:0}$, the major fatty acid in members of the genus *Paenibacillus*, was also the major fatty acid component (36.59%) of strain T27^T.

In conclusion, on the basis of the phenotypic properties, 16S rRNA gene sequences, G+C contents, levels of DNA–DNA hybridization, chemotaxonomic properties and *nifH* sequences, the five novel strains form a very homogeneous

Table 2. Characteristics of *P. sabinae* sp. nov. and closely related *Paenibacillus* species

Taxa: 1, *P. sabinae* (strains T27^T, T49, T67, JD2 and G18-7); 2, *P. azotofixans* ATCC 35681^T; 3, *P. polymyxa* DSM 36^T; 4, *P. macerans* ATCC 8244^T; 5, *P. odorifer* TOD45^T; 6, *P. graminis* RSA19^T; 7, *P. wynnii* LMG 22176^T; 8, *Paenibacillus stellifer* DSM 14472^T; 9, *P. brasiliensis* DSM 14914^T; 10, *P. peoriae* IFO 15541^T. All show anaerobic growth. +, Positive reaction; –, negative reaction; v, variable reaction; ND, not determined.

Characteristic	1	2	3	4	5*	6*	7†	8‡	9*	10*
Optimum growth temp. (°C)	30	30	30	30	30	30	20	28	30	30
Growth in/with:										
3 % NaCl	+	+	+	+	ND	ND	–	ND	ND	ND
5 % NaCl	–	–	–	–	–	ND	–	ND	–	–
0.001 % lysozyme	–	–	–	+	+	–	ND	ND	+	+
Catalase	+	+	+	+	+	+	+	ND	ND	+
Oxidase	–	–	–	–	–	–	–	–	ND	–
Methyl red	+	+	+	+	+	+	ND	ND	ND	ND
Voges–Proskauer	+	+	+	+	–	–	–	ND	+	+
Nitrate reduction	+	+	+	+	+	+	+	–	+	+
Production of:										
Dihydroxyacetone	–	–	–	–	–	–	–	ND	–	–
Indole	–	–	–	–	–	–	–	ND	ND	–
Dextrin	–	–	–	+	–	–	–	+	–	–
Hydrolysis of:										
Casein	–	–	–	–	–	–	–	ND	+	+
Gelatin	–	–	–	–	–	–	–	ND	v	+
Starch	–	–	–	–	–	–	+	+	+	+
Decomposition of tyrosine	–	–	–	–	–	–	–	ND	ND	–
Production of acid from:										
Sucrose	–	–	+	+	+	–	+	+	ND	+
Glucose	–	+	+	+	+	+	+	+	+	ND
Glycerol	–	–	+	+	–	–	v	–	–	v
Maltose	–	+	+	+	+	+	+	+	ND	ND
Lactose	–	–	+	+	ND	ND	+	+	+	+
D-Sorbitol	–	–	+	+	–	–	v	–	–	+
Sodium succinate	–	–	–	+	–	–	ND	–	+	+
Sodium citrate	–	–	–	–	–	–	ND	–	+	+
D-Xylose	–	–	–	–	–	–	+	+	–	+
Fructose	–	–	–	–	–	–	+	+	+	–
Glycine	–	–	–	–	–	–	ND	ND	ND	ND
L-Aspartate	–	+	+	+	+	+	ND	–	ND	ND
DNA G + C content (mol%)	51.9–52.9	50.7	45	53	44	52.1	44.6	55.6	ND	46

*Data from von der Weid *et al.* (2002).

†Data from Rodríguez-Díaz *et al.* (2005).

‡Data from Suominen *et al.* (2003).

group that is distinct from other related species within the genus *Paenibacillus*. Therefore, we propose that they represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus sabinae* sp. nov. is proposed.

Description of *Paenibacillus sabinae* sp. nov.

Paenibacillus sabinae (sa'bi.nae. N.L. gen. n. *sabinae* of Sabina, referring to the plant *Sabina squamata*, the source of the rhizosphere soil from which the type strain was isolated).

Cells are Gram-positive, aerobic, motile, straight rods (0.7–0.8 × 2.7–3.2 µm). Oval or ellipsoidal endospores are produced, located subterminally or centrally in swollen sporangia. Colonies on nutrient agar are circular, convex and glossy with entire margins. The temperature range for growth is 4–37 °C (optimum, 30 °C). Grows at pH 4.2–10.0 (optimum, pH 7.2). Grows in 3 % (w/v) NaCl, but does not tolerate NaCl at 5 % (w/v). Does not grow in 0.001 % (w/v) lysozyme. Catalase-positive and oxidase-negative. Voges–Proskauer and methyl red reactions are positive. Nitrate is

Table 3. Cellular fatty acid profiles (%) of *P. sabinae* sp. nov. T27^T and closely related *Paenibacillus* species

Strains: 1, strain T27^T; 2, *P. azotofixans* ATCC 35681^T (data from 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.

Fatty acid	1	2	3	4	5
Saturated straight-chain					
C _{14:0}	2.96	5.0	0.7	5.0	3.7
C _{15:0}	1.54	2.2	0.5	3.4	0.5
C _{16:0}	18.19	15.5	9.1	20.5	17.9
C _{17:0}	0.71	NR	NR	NR	NR
C _{18:0}	3.28	0.3	1.1	0.3	NR
Saturated iso-branched					
C _{13:0} 3-OH	1.39	NR	NR	NR	NR
C _{14:0}	3.20	4.7	0.6	4.7	1.8
C _{15:0}	1.37	8.7	5.5	8.7	2.6
C _{15:1} I	0.71	NR	NR	NR	NR
C _{16:0}	14.40	5.3	7.7	NR	17.1
C _{17:0}	0.79	1.1	7.0	NR	4.1
Saturated anteiso-branched					
C _{15:0}	36.59	45.4	49.9	45.5	34.5
C _{17:0}	6.38	2.1	16.7	2.1	16.1
Unsaturated*					
C _{16:1} ω7c	1.78	NR	NR	NR	NR
C _{18:1} ω7c	1.22	NR	NR	NR	NR
C _{18:1} ω9c	3.88	<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*.

reduced to nitrite. Nitrogen fixation is detected by acetylene reduction and from the presence of the *nifH* gene. Acid and gas are not produced from the following carbohydrates: glucose, sucrose, lactose, fructose, glycerol, D-xylose, maltose, D-sorbitol, sodium succinate, sodium citrate, glycine and L-aspartate. Gelatin, casein and starch are not hydrolysed. The major cellular fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and C_{16:0}.

The DNA G+C content of the type strain is 51.9%. The type strain, T27^T (=CCBAU 10202^T=DSM 17841^T), was isolated from the rhizosphere soil of *Sabina squamata* planted in Beijing, China.

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