Paenibacillus elgii sp. nov., with broad antimicrobial activity

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Two novel spore-forming bacteria with broad antimicrobial activity were isolated from roots of *Perilla frutescens*. The isolates, SD17^T and SD18, were facultatively anaerobic and showed variable Gram reaction. Growth was observed between 20 and 45 °C. DNA G + C content of SD17^T was 51.7 mol%, and the major fatty acid was anteiso-C_{15:0} (54.1 %). 16S rRNA gene sequence similarity of SD17^T ranged from 98.6 to 91.3 % with other *Paenibacillus* species. The phylogenetic tree showed that isolate SD17^T formed a significant monophyletic clade with *Paenibacillus koreensis* KCTC 2393^T and *Paenibacillus ehimensis* IFO 15659^T. DNA–DNA relatedness values for strain SD17^T with *Paenibacillus koreensis* KCTC 2393^T and *Paenibacillus ehimensis* IFO 15659^T were 17.4 and 19.8 %, respectively. These isolates thus merit species status within *Paenibacillus*, for which the name *Paenibacillus elgii* sp. nov. is proposed. The type strain is SD17^T (=KCTC 10016BP^T=NBRC 100335^T).

The genus *Paenibacillus* was proposed by Ash *et al.* (1993) on the basis of polyphasic data from 16S rRNA gene sequences, and then emended by Shida *et al.* (1997). Members of the genus *Paenibacillus* are facultatively anaerobic or strictly aerobic, rod-shaped, produce ellipsoidal spores in swollen sporangia and have G + C contents ranging from 45 to 54 mol% (Ash *et al.*, 1993). Some of these organisms excrete diverse assortments of polysaccharide-hydrolysing enzymes (Kanzawa *et al.*, 1995; Nakamura, 1987; Priest *et al.*, 1988) and produce antibacterial compounds such as polymyxin, octopytin and baciphelacin (Slepecky & Hemphill, 1991) and an antifungal compound (Chung *et al.*, 2000).

The taxonomic classification of two isolates, SD17^T and SD18, was studied here based on phylogenetic and phenotypic evidence. We propose that the two isolates should be classified in the genus *Paenibacillus* as *Paenibacillus elgii* sp. nov.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SD17^T and SD18 are AY090110 and AY081186, respectively.

Micrographs and an expanded phylogenetic tree are available as supplementary material in IJSEM Online.

Strains SD17^T and SD18 were isolated from roots of *Perilla frutescens* collected from Seochon, Korea. The roots were washed under running tap water, heat-treated at 80 °C for 30 min, macerated and plated onto 1 : 10 diluted trypticase soy agar (1/10 TSA; Difco) for isolation of single colonies similar to the method described by Kim *et al.* (1997). These isolates were examined to screen for inhibitory activity against growth of *Rhizoctonia solani* AG 4 and *Pythium aphanidermatum*. Of approximately 3000 selected candidates, the isolates that suppressed diseases of turfgrass (*Agrostis palustris*) caused by *Pythium aphanidermatum* (pythium blight) and *R. solani* AG 2-2 (brown patch) were selected for further studies. Isolates SD17^T and SD18 were inhibitory to the growth of various fungi such as *Botrytis cinerea*, *Chaetomium globosum*, *Cladosporium resinae*, *Colletotrichum gloeosporioides*, *Corynespora cassicola*, *Fusarium oxysporum* f. sp. *lycopersici*, *Magnaporthe grisea*, *Phytophthora infestans*, *Pythium aphanidermatum*, *R. solani* AG 1-1, *R. solani* AG 2-2, *R. solani* AG 4, *Saccharomyces cerevisiae*, *Sclerotinia homoeocarpa* and *Trichoderma viride* and bacteria including *Bacillus subtilis*, *Burkholderia glumae*, *Escherichia coli*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens*, *Xanthomonas oryzae* and *Xanthomonas vesicatoria*, when extensively tested for their antimicrobial activity. Strains SD17^T and SD18 were routinely cultured on 1/10 TSA and maintained in 20 % glycerol at –80 °C for storage.

Cell morphology was examined using a Nikon MICROPHOT-FXA phase-contrast microscope. The cells of 24 h cultures were Gram-stained using Hucker's modification (Gerhardt *et al.*, 1994). For electron microscopy, vegetative cells were negatively stained with 1% (w/v) phosphotungstic acid and, after air-drying, the grids were examined under a Philips CM-20 transmission electron microscope. Spores were pre-fixed in 5% (v/v) glutaraldehyde in phosphate buffer (Gibco) for at least 1 h at room temperature. Samples were then post-fixed in 4% (w/v) osmium tetroxide for 1 h, dehydrated using a graded series of acetone, transferred to 100% acetone and embedded in Epon 812 (Fluka) substitute. Thin sections were cut with a diamond knife on a Leica Ultracut VCT microtome and stained with uranyl acetate and lead citrate. Grids were examined on a Philips CM-20 transmission electron microscope at an operating voltage of 60 kV. Phenotypic characterization was carried out using the standard methods of Claus & Berkeley (1986) and McFaddin (2000), together with API 20E and API 50CHB systems (bioMérieux). Oxidation of 95 selected carbon sources was tested using Biolog GP2 microplates according to the manufacturer's instructions. Growth was examined using a cap tube containing 10 ml tryptic soy broth, 100 mM Na₂HPO₄/NaH₂PO₄ buffer, with a pH of 6.0–9.0 at 30 °C. Growth was estimated by monitoring the optical density at 650 nm.

Table 1. Distinctive phenotypic characteristics of *Paenibacillus elgii* sp. nov. and closely related strains

Species: 1, *Paenibacillus elgii* sp. nov. (*n*=2); 2, *Paenibacillus ehimensis* IFO 15659^T (data from Kuroshima *et al.*, 1996); 3, *Paenibacillus koreensis* KCTC 2393^T (Chung *et al.*, 2000); 4, *Paenibacillus azoreducens* DSM 13822^T (Meehan *et al.*, 2001). +, Positive; –, negative; v, variable; NT, not tested. All species are positive for catalase, hydrolysis of starch and fermentation of mannitol and are negative for production of acetylmethylcarbinol and utilization of citrate.

Characteristic	1	2	3	4
Anaerobic growth	+	–	+	+
Oxidase	–	+	+	–
Nitrate reduction	+	+	+	–
Production of indole	+	NT	–	–
Hydrolysis of casein	+	+	+	–
Growth at pH 5.6	+	NT	+	+
Growth at 50 °C	–	+	+	+
Growth in 5% NaCl	–	NT	+	–
Fermentation of:				
L-Arabinose	–	+	+	–
D-Xylose	v	+	–	+
Glycerol	v	NT	NT	+
DNA G+C content (mol%)	51.7*	52–55	54	47

*Value for strain SD17^T.

The cells are Gram-variable and exhibit motility by peritrichous flagella. Ellipsoidal spores formed in swollen sporangia and mature spores have surface stripes. They exhibit a star-shaped morphology in thin section when visualized by transmission electron microscopy (Elo *et al.*, 2001; see Supplementary Figs A and B, available in IJSEM Online). Distinguishing phenotypic characteristics between the isolates and phylogenetically related species are shown in Table 1.

Whole-cell fatty acids were analysed as described by Lee *et al.* (1996). Menaquinones were analysed by reversed-phase HPLC as described by Komagata & Suzuki (1987). The diamino acid of the peptidoglycan was determined by TLC (DC-Alufoline cellulose; Merck) as described by Komagata & Suzuki (1987). G+C content of the DNA was determined from the midpoint value of the thermal denaturation profile using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech) equipped with a programmable Peltier temperature control unit according to the equation of Marmur & Doty (1962) as modified by De Ley (1970). Strains SD17^T and SD18 contained MK-7 as the major menaquinone and *meso*-diaminopimelic acid in the cell-wall peptidoglycan. These chemotaxonomic characteristics were most similar to the those characteristic of the genus *Paenibacillus* (Shida *et al.*, 1997). The DNA G+C content of strain SD17^T was 51.7 mol%. Whole-cell fatty acid compositions of the two isolates and related species are shown in Table 2. According to the published data, the cellular fatty acid pattern of strains SD17^T and SD18 is similar to those of phylogenetically closely related

Table 2. Whole-cell fatty acid compositions of *Paenibacillus elgii* sp. nov. and closely related strains

Strains: 1, *Paenibacillus elgii* SD17^T; 2, *Paenibacillus elgii* SD18; 3, *Paenibacillus ehimensis* IFO 15659^T (data from Lee *et al.*, 2004); 4, *Paenibacillus koreensis* KCTC 2393^T (Lee *et al.*, 2004); 5, *Paenibacillus azoreducens* DSM 13822^T (Meehan *et al.*, 2001). ND, Not detected.

Fatty acid	1	2	3	4	5
Saturated					
C _{14:0}	1.9	1.0	1.0	1.2	3.5
C _{15:0}	1.2	1.0	1.2	0.8	0.1
C _{16:0}	9.7	6.2	7.1	8.5	22.1
Branched					
iso-C _{14:0}	2.1	1.3	1.8	1.6	0.8
iso-C _{15:0}	10.6	13.7	8.1	6.0	5.9
anteiso-C _{15:0}	54.1	62.0	52.9	43.4	33.9
iso-C _{16:0}	6.5	3.6	8.6	12.2	8.6
iso-C _{17:0}	3.2	2.6	3.3	5.5	5.6
anteiso-C _{17:0}	6.2	6.1	8.0	11.9	19.8
Unsaturated					
C _{16:1} ω7c alcohol	ND	ND	1.2	1.4	ND
C _{16:1} ω11c	4.5	2.5	5.2	5.4	ND

species of the genus *Paenibacillus*. The main fatty acid is anteiso- $C_{15:0}$, comprising 54–62 % of the total. These values are similar to *Paenibacillus ehimensis*, but higher than those reported for *Paenibacillus koreensis* KCTC 2393^T (Lee *et al.*, 2004) and *Paenibacillus azoreducens* DSM 13822^T (Meehan *et al.*, 2001). The second major fatty acid in strains SD17^T and SD18 is iso- $C_{15:0}$, whereas it is iso- $C_{16:0}$ in *Paenibacillus ehimensis* and *Paenibacillus koreensis*. In *Paenibacillus azoreducens*, saturated fatty acid $C_{16:0}$ was the second major component. This clearly distinguished the isolates from related species.

The primary structure of the 16S rRNA gene was determined as described by Chun & Goodfellow (1995). The resultant sequence of strain SD17^T was manually aligned with representative sequences of *Paenibacillus* obtained from the GenBank database, using known 16S rRNA secondary structure information (Gutell, 1994). Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967) and neighbour-joining methods (Saitou & Nei, 1987). Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant neighbour-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The almost complete 16S rRNA gene sequence (1481 bp) of SD17^T was determined and its primary structure was compared with closely related reference strains. A phylogenetic tree based on the nucleotide substitution rate (K_{nuc} values) indicates that the strains belong to the genus *Paenibacillus* (Fig. 1). When the phylogenetic position of isolates SD17^T and SD18 was compared with closely related species of the genus *Paenibacillus*, the strains formed a monophyletic clade with *Paenibacillus koreensis* KCTC 2393^T and *Paenibacillus ehimensis* IFO 15659^T (see Supplementary Fig. C). The nucleotide sequence similarity values between SD17^T and other *Paenibacillus* species ranged from 98.6 % (with *Paenibacillus ehimensis* IFO 15659^T) to 91.3 % (with *Paenibacillus kobensis* IFO 15729^T). The next most closely related species is *Paenibacillus koreensis*, with 97.9 % sequence similarity. Therefore, the genealogical position of strains SD17^T and SD18 was further examined using DNA–DNA relatedness with *Paenibacillus ehimensis* IFO 15659^T and *Paenibacillus koreensis* KCTC 2393^T.

Bacillus ehimensis and *Bacillus chitinolyticus*, both of which exhibit chitinolytic activity, were first described by Kuroshima *et al.* (1996). At that time, they studied the morphological and biochemical characteristics of *Bacillus ehimensis* and *Bacillus chitinolyticus*, yet did not describe their phylogenetic relationship with related taxa. Recently, the taxonomic status of *Bacillus ehimensis* and *Bacillus chitinolyticus* was examined based on 16S rRNA gene sequences, DNA–DNA hybridization and other taxonomic characteristics (Lee *et al.*, 2004). The study reported that the two species belonged to the genus *Paenibacillus* and

Fig. 1. Phylogenetic tree showing the position of *Paenibacillus elgii* sp. nov. SD17^T with other species of the genus *Paenibacillus* and related taxa based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are indicated. *Alicyclobacillus acidocaldarius* was used as an outgroup. Bar, 0.05 accumulated changes per nucleotide.

proposed that *Bacillus ehimensis* and *Bacillus chitinolyticus* be transferred to the genus *Paenibacillus* as *Paenibacillus ehimensis* comb. nov. and *Paenibacillus chitinolyticus* comb. nov.

DNA–DNA hybridization was determined based on a membrane filter technique using a DIG High Prime DNA labelling and detection starter kit II (Roche Molecular Biochemicals). Genomic DNA (200 ng) was denatured by the alkaline method and immobilized on a nylon membrane (Hybond-N⁺; Amersham) by applying a low vacuum and the DNA preparations (1 µg) were labelled using the DIG High Prime DNA labelling and detection starter kit II according to the manufacturer's protocol. The membranes were then pre-hybridized in a hybridization solution at 52 °C for 30 min. The actual pre-hybridization was carried out in a hybridization solution containing labelled DNA (25 ng ml⁻¹) at 52 °C for 16 h. After hybridization, the membranes were washed twice in a primary washing solution (2 × SSC and 0.1 % SDS) and then subsequently washed twice in a secondary solution (0.5 × SSC and 0.1 % SDS) at 68 °C. Detection reagents were added to the membranes for 5 min at room temperature, the membranes were then exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 10 min and the signal intensities were determined using the TINA 2.0 program. The signal produced by self-hybridization of the probe with homologous target DNA was taken as 100 %, and percentage relatedness values were calculated for the duplicate samples.

DNA–DNA hybridization results confirmed that isolates SD17^T and SD18 belonged to the same species. Relatedness between the two strains was 89.5 %. Strain SD17^T showed 19.8 and 17.4 % DNA–DNA relatedness with *Paenibacillus ehimensis* IFO 15659^T and *Paenibacillus koreensis* KCTC 2393^T, respectively. The latter two strains showed 26 % mutual DNA–DNA relatedness (Lee *et al.*, 2004). DNA–DNA relatedness of these strains with strain SD18 was also very low (17.2 % with *Paenibacillus ehimensis* IFO 15659^T and 15.0 % with *Paenibacillus koreensis* KCTC 2393^T).

The phylogenetic definition of a species of Wayne *et al.* (1987) includes strains with approximately 70 % or more DNA–DNA relatedness. Organisms that have less than 97 % 16S rRNA gene sequence similarity will not reassociate to more than 60 %, no matter which hybridization method is applied (Stackebrandt & Goebel, 1994). We did not determine the DNA–DNA hybridization value with *Paenibacillus azoreducens* because of low 16S rRNA gene sequence similarity (94 %). This result, together with phylogenetic analysis, demonstrates that isolates SD17^T and SD18 are distinct from all previously described *Paenibacillus* taxa at the species level.

On the basis of morphological, physiological, chemotaxonomic characteristics, 16S rRNA gene sequence analysis and DNA–DNA relatedness, strains SD17^T and SD18 represent a novel species of the genus *Paenibacillus* for which the name *Paenibacillus elgii* sp. nov. is proposed.

Description of *Paenibacillus elgii* sp. nov.

Paenibacillus elgii (el'gi.i. N.L. gen. n. *elgii* arbitrary name formed from the company name LG where taxonomic studies on this species were performed).

Cells are facultatively anaerobic, Gram-variable rods (0.8–1.0 µm wide, 3.0–5.0 µm long), motile with peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia and mature spores have stripes on the surface. Colonies on nutrient agar are circular, flat, smooth, opaque and white. Temperature range for growth is 20–45 °C; growth occurs at pH 6.0–8.5 (optimum 7.0). Isolates are able to grow in the presence of 2 % NaCl. Catalase-positive, oxidase-negative. Reduction of nitrate is positive. H₂S is not produced but indole is produced. Casein, aesculin and starch are hydrolysed. Acid is produced from glucose, maltose, mannitol, mannose and trehalose. Glucose, ribose, *N*-acetylglutamate and Tween 40 are assimilated. The G+C content of the DNA is 51.7 mol%. The major isoprenoid quinone is menaquinone MK-7. The major cellular fatty acid is anteiso-C_{15:0}. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid.

The type strain is SD17^T (=KCTC 10016BP^T=NBRC 100335^T) and SD18 (=KCTC 3756) is a reference strain. Isolated from roots of *Perilla frutescens* in Seochon County, Korea.

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