Fontibacillus panacisegetis sp. nov., isolated from soil of a ginseng field

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A facultatively anaerobic, endospore-forming bacterium, designated strain P11-6^T, was isolated from soil of a ginseng field located in Geumsan County, Republic of Korea. Cells of strain P11-6^T were Gram-stain-negative, catalase-negative, motile rods and produced semi-translucent, circular, white colonies on tryptic soy agar. The isolate contained MK-7 as the only menaquinone and anteiso-C_{15:0} as the major fatty acid. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an unknown aminophosphoglycolipid, an unknown aminophospholipid, two unknown phospholipids, three unknown glycolipids and three unknown lipids were detected in the polar lipid profile. The DNA G + C content of strain P11-6^T was 41.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing showed that strain P11-6^T was most closely related to *Fontibacillus aquaticus* GPTSA 19^T (97.2 % sequence similarity) and that it formed a separate lineage with *F. aquaticus* in the family *Paenibacillaceae*. Combined phenotypic and DNA–DNA hybridization data supported the conclusion that strain P11-6^T represents a novel species in the genus *Fontibacillus*, for which the name *Fontibacillus panacisegetis* sp. nov. is proposed; the type strain is P11-6^T (=KCTC 13564^T =CECT 7605^T).

The family *Paenibacillaceae* of the order *Bacillales* currently comprises nine recognized genera, *Ammoniphilus*, *Aneurinibacillus*, *Brevibacillus*, *Cohnella*, *Fontibacillus*, *Oxalophagus*, *Paenibacillus*, *Saccharibacillus* and *Thermobacillus*, which accommodate Gram-stain-positive, -variable or -negative, sporulating bacilli. The genus *Paenibacillus* (type species, *Paenibacillus polymyxa*) was created by Ash *et al.* (1993) to accommodate the so-called rRNA group 3 bacilli comprising 11 *Bacillus* species on the basis of comparative 16S rRNA gene sequence analysis. Since then, ten more *Bacillus* species and one *Clostridium* species have been transferred to this genus and a huge number of novel species have been described. Thus, at the time of writing, the genus *Paenibacillus* contained 109 recognized species (Euzéby, 2010). Most recently, the genus *Fontibacillus* has been described on the basis of its 16S rRNA gene dissimilarity with respect to related species of the genus

Paenibacillus and its unique polar lipid profile, despite its phylogenetic position within the large *Paenibacillus* cluster (Saha *et al.*, 2010).

Strain P11-6^T was recovered during the course of an investigation of the culturable aerobic and facultatively anaerobic bacterial community in soil from a ginseng field. At first, the strain was considered to belong to the genus *Paenibacillus* on the basis of preliminary 16S rRNA gene sequence comparison, although it showed low levels of similarity (<96 %) to all recognized species of this genus. However, after the description of the novel genus *Fontibacillus*, strain P11-6^T was found to show a higher 16S rRNA gene sequence similarity (>97 %) to *Fontibacillus aquaticus* GPTSA 19^T, the only member of this genus. Thus, a polyphasic taxonomic investigation was carried out to clarify the taxonomic status of strain P11-6^T.

Strain P11-6^T was isolated from a soil sample collected from a ginseng field located in Geumsan County, Republic of Korea, by the dilution plate technique. The soil sample was diluted serially in sterile distilled water and samples of each serial dilution were spread on one-tenth-strength R2A agar (Difco) and incubated at 25 °C for 4 weeks. A pinpoint colony showing delayed growth was isolated. The isolate was purified by subculturing on the same medium and maintained as glycerol/water suspensions (20 %, v/v) at –70 °C. Due to the delayed growth on the

Abbreviations: AL, unknown aminolipid; APGL, unknown aminophosphoglycolipid; DPG, diphosphatidylglycerol; GL, unknown glycolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unknown phospholipid; PN, unknown aminophospholipid; UL, unknown lipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Fontibacillus panacisegetis* P11-6^T (=KCTC 13564^T) is GQ303568.

Two supplementary figures and a supplementary table are available with the online version of this paper.

above medium, the isolate was cultivated on various complex media. Subsequently, it was grown routinely on solid rich medium (R medium; Yamada & Komagata, 1972). For most experiments, strain P11-6^T was cultivated on tryptic soy agar (TSA) or broth (TSB; Difco) at 25 °C for 48 h, unless stated otherwise. For comparative purposes, *F. aquaticus* DSM 17643^T, *Paenibacillus anaericanus* DSM 15890^T and *Paenibacillus ginsengisoli* KCTC 13931^T were grown on the same medium at 30 °C for 48 h.

The Gram reaction was performed as described by Smibert & Krieg (1994). Cell morphology and motility were observed under a phase-contrast microscope (Eclipse 80i; Nikon). Motility was tested using the hanging-drop technique (Skerman, 1967). For the observation of flagella and endospores, cells were negatively stained with 1% (w/v) uranyl acetate and examined using a model H-7600 transmission electron microscope (Hitachi). For the formation of endospores, cells were grown on nutrient sporulation medium (Schaeffer *et al.*, 1965) at 25 °C for 7 days and thin sections of cells were prepared as described by Paster & Canale-Parola (1982). Oxidase activity was tested by using the oxidase reagent (bioMérieux) and catalase activity was determined by production of bubbles after the addition of a drop of 3% H₂O₂. Growth was investigated at 5, 10, 15, 20, 25, 30, 37, 42 and 45 °C, in the presence of 1, 2, 3, 5, 6.5 and 10% NaCl, and at pH 5–11 (in increments of 0.5 pH units). The following biological buffers were used to adjust the pH: Na₂HPO₄/NaH₂PO₄ for pH below 9 and Na₂CO₃/NaHCO₃ for pH 9–11 (Gomori, 1955). Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber (H₂/CO₂/N₂, 7:5:88, by vol.; Thermo Scientific) on TSA or on TSA supplemented with 0.1% (w/v) KNO₃. Hydrolysis of casein, DNA, starch and tyrosine was tested on DNase test agar, casein agar, starch agar and tyrosine agar (BBL), respectively. Hydrolysis of Tweens 20 and 80 was examined by using the method of Sierra (1957). The production of lecithinase was detected on egg yolk agar (McClung & Toabe, 1947). H₂S production was tested in TSB supplemented with 0.01% (w/v) cysteine, with a strip of lead acetate paper (Fluka) as an indicator. The methyl red and Voges–Proskauer tests were performed in MR-VP broth (BBL). Single carbon source assimilation tests were performed in mineral salts basal medium (Stanier *et al.*, 1966) supplemented with 1% (v/v) vitamin solution (Staley, 1968). Filter-sterilized substrate (0.1%, w/v) was added to this medium, with the exception of carbohydrates, which were used at 0.2% (w/v). The organic compounds used as sole sources of carbon and energy were acetate, *N*-acetylglucosamine, *L*-alanine, amygdalin, *L*-arabinose, *L*-arabitol, cellobiose, citrate, *D*-fructose, *L*-fucose, *D*-galactose, gentiobiose, gluconate, *D*-glucose, glycerol, glycogen, *L*-histidine, inositol, *DL*-lactate, lactose, malate, malonate, maltose, *D*-mannitol, *D*-mannose, melizitose, melibiose, *L*-proline, propionate, raffinose, *L*-rhamnose, *D*-ribose, *D*-salicin, *L*-serine, *D*-sorbitol, starch, sucrose, trehalose, turanose and *D*-xylose. Acid production

tests, enzyme activity tests and additional phenotypic tests were performed using API 50CH (with CHB/E medium), API ZYM, API 20E and API 20NE galleries according to the instructions of the manufacturer (bioMérieux). Duplicate antibiotic-susceptibility tests were performed using SensiDiscs (6 mm; BBL) containing amikacin (30 µg), bacitracin (10 IU), ceftazidime (30 µg), ceftriaxone (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), colistin (10 µg), doxycycline (30 µg), gentamicin (10 µg), imipenem (10 µg), norfloxacin (10 µg), novobiocin (30 µg), oxacillin (1 µg), polymyxin B (300 IU) and tobramycin (10 µg); zone diameter interpretive standards for staphylococci by the NCCLS (2003) were used for interpretation.

For analysis of fatty acids, cells were grown on TSA at 25 °C for 3 days. Fatty acid methyl esters were prepared and analysed as described by Klatte *et al.* (1994) by using the standard Microbial Identification System (MIDI) for automated GC analysis (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and purified by using TLC on Kieselgel 60 F₂₅₄ plates (20 × 20 cm, 0.5 mm thickness; Merck) with petroleum ether/diethyl ether (9:1, v/v) as the solvent. Quinones were identified by using reversed-phase HPLC as described by Shin *et al.* (1996). Amino acid and sugar analyses of whole-cell hydrolysates followed the procedures of Stanek & Roberts (1974). Polar lipids were extracted, examined by two-dimensional TLC and identified by using published procedures (Minnikin *et al.*, 1977).

Genomic DNA was extracted using an UltraClean microbial DNA isolation kit (Mo Bio Laboratories). The 16S rRNA gene was amplified from chromosomal DNA using a universal bacterial primer set (27F and 1492R; Lane, 1991). The amplified PCR product was purified using a PCR purification kit (Solgent) and sequenced directly by the fluorescent dye terminator method using an ABI 3730XL capillary DNA analyser (Applied Biosystems) at Solgent (Daejeon, Korea). The full-length 16S rRNA gene sequence was compiled using the SeqMan software (DNASTAR) and aligned with published sequences retrieved from GenBank/EMBL/DBJ using CLUSTAL X (Thompson *et al.*, 1997); the resulting multiple alignment was edited manually using the program BioEdit (Hall, 1999). The neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms were used in phylogenetic tree-building. Phylogenetic dendrograms were reconstructed by using the DNADIST, NEIGHBOR, DNAPARS and DNAML programs in the PHYLIP package (Felsenstein, 2009). The distance matrix was produced on the basis of the Jukes–Cantor model (Jukes & Cantor, 1969). Bootstrap analysis (Felsenstein, 1985) was done with 1000 replicates except in the maximum-likelihood algorithm, where only 100 replicates were generated. For the measurement of G + C content, genomic DNA was extracted and purified using a Blood & Cell Culture DNA Midi kit (Qiagen). The DNA was degraded enzymically into nucleosides and G + C content was determined by using reversed-phase HPLC, as described

by Tamaoka & Komagata (1984). DNA–DNA hybridization was performed fluorometrically (Ezaki *et al.*, 1989) by using DNA probes labelled with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner Bio-One) at 42 °C.

Strain P11-6^T formed visible colonies (about 1 mm in diameter) either within 48 h on TSA incubated at 25 °C or within 3 days on solid R medium incubated at 25 °C. Good growth occurred at temperatures ranging from 20 to 30 °C. Colonies were white, slightly convex, semi-translucent, smooth and circular with entire edges on TSA, whereas they were white, flat, opaque, rough and slightly irregular on solid R medium. Cells were Gram-stain-negative, facultatively anaerobic, motile rods with peritrichous flagella. Terminal to subterminal, ellipsoidal spores were formed in swollen sporangia (Supplementary Fig. S1, available in IJSEM Online). Other physiological, biochemical and chemotaxonomic characteristics are summarized in the species description.

The almost-complete 16S rRNA gene sequence (1445 nt) of strain P11-6^T was determined and compared with those of all known species of the family *Paenibacillaceae*. Strain P11-6^T showed highest 16S rRNA gene sequence similarity (97.2 %) to *F. aquaticus* GPTSA 19^T and showed the next

highest similarity (95.7 %) to both *P. anaericanus* MH21^T and *P. ginsengisoli* Gsoil 1638^T. In the phylogenetic tree, strain P11-6^T occupied a distinct position, clustering only with *F. aquaticus* GPTSA 19^T (Fig. 1). The clade composed of strain P11-6^T and *F. aquaticus* GPTSA 19^T was supported by all three tree-making algorithms (neighbour-joining, maximum-parsimony and maximum-likelihood) and by bootstrap values greater than 70 %, whereas it did not form any stable phylogenetic cluster with nearby *Paenibacillus* species, which supported the affiliation of strain P11-6^T to the genus *Fontibacillus*.

The physiological and biochemical characteristics that serve to differentiate strain P11-6^T from its closest phylogenetic neighbours are listed in Table 1. Strain P11-6^T showed significant differences in terms of catalase activity, growth at 42 °C, growth in 3 % NaCl, acid production from trehalose, utilization of D-fructose, gentiobiose, glycerol and trehalose, esterase lipase (C8) and α -galactosidase activities, hydrolysis of Tween 20, and susceptibility to colistin and norfloxacin with respect to related type strains of the genus *Paenibacillus*, which were shared with the type strain of *F. aquaticus*. However, strain P11-6^T could be clearly distinguished from the type strain of *F. aquaticus* by differences in the methyl red test result, nitrate reduction, lecithinase activity, acid from D-ribose,

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, reconstructed with the maximum-likelihood method, showing the position of strain P11-6^T among related taxa within the family *Paenibacillaceae*. Numbers at nodes are levels of bootstrap support (≥ 50 %) based on 100 resamplings. Filled circles indicate nodes that are also found with the neighbour-joining and maximum-parsimony algorithms. *Lactobacillus delbrueckii* subsp. *lactis* ATCC 12315^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 1. Physiological and biochemical characteristics that differentiate strain P11-6^T from the type strains of related taxa

Strains: 1, *Fontibacillus panacisegetis* sp. nov. P11-6^T; 2, *Fontibacillus aquaticus* DSM 17643^T; 3, *Paenibacillus anaericanus* DSM 15890^T; 4, *Paenibacillus ginsengisoli* KCTC 13931^T. +, Positive; (+), weakly positive; –, negative. All strains tested were positive for motility, oxidase activity, anaerobic growth and the Voges–Proskauer test. All strains tested were negative for Gram staining and H₂S production.

Characteristic	1	2	3	4
Catalase	–	–	+	+
Growth at/with:				
42 °C	+	+	–	–
3 % NaCl	–	–	+	+
Methyl red test	–	+	–	–
Nitrate reduction	+	–	+	+
Lecithinase on egg yolk agar	–	+	–	–
Acid production from:				
D-Ribose	–	+	+	+
Trehalose	–	–	+	+
Utilization of:				
N-Acetylglucosamine	–	+	+	(+)
Amygdalin	–	+	+	+
D-Fructose	+	+	–	–
Gentiobiose	+	(+)	–	–
Glycerol	+	+	–	–
Raffinose	–	+	–	–
D-Ribose	–	+	+	+
Trehalose	–	–	+	+
Hydrolysis of:				
Casein	(+)	–	–	–
DNA	+	–	–	–
Starch	–	(+)	(+)	+
Tween 20	(+)	(+)	–	–
Susceptibility to:				
Bacitracin	–	+	+	+
Colistin	+	+	–	–
Norfloxacin	–	–	+	+
Enzyme activity:*				
Esterase (C4)	–	+	+	+
Esterase lipase (C8)	–	–	+	+
Leucine arylamidase	+	–	+	+
α-Chymotrypsin	+	–	–	–
α-Galactosidase	+	+	–	–

*Data from API ZYM tests.

utilization of *N*-acetylglucosamine, amygdalin, raffinose and D-ribose, hydrolysis of casein, DNA and starch, susceptibility to bacitracin, and the presence of esterase (C4), leucine arylamidase and α-chymotrypsin activities.

The fatty acid profile of strain P11-6^T was similar to those of related taxa, i.e. almost all components were common in all taxa and the fatty acid anteiso-C_{15:0} was predominant. The only difference was that the relative amount of C_{16:0} was so low that it could not be a major component. Detailed fatty acid compositions are shown in Table 2. The

polar lipid profile of strain P11-6^T was also similar to that of the type strain of *F. aquaticus* except that strain P11-6^T lacked the minor components unknown phospholipid (PL) 7, PL8, PL9, PL12, unknown aminolipid (AL) 2 and AL3 and also lacked unknown lipid (UL) 6, but did have UL8. In contrast, the polar lipid profiles of strain P11-6^T and the type strain of *F. aquaticus* showed significant differences from that of the type strain of *Paenibacillus polymyxa* (the type species of the genus *Paenibacillus*), e.g. the presence of unknown aminophosphoglycolipid (APGL), unknown aminophospholipid (PN) 2, PL2, unknown glycolipid (GL) 1, GL3 and UL2 and the absence of PN1, AL1, GL2 and UL7 (Supplementary Fig. S2 and Supplementary Table S1).

On the basis of 16S rRNA gene sequence similarity values, together with its phylogenetic position, and its polar lipid profile, strain P11-6^T could be classified as a member of the genus *Fontibacillus*. The DNA–DNA hybridization value of 23 % between strain P11-6^T and the type strain of *F. aquaticus* and the phenotypic differences between them support the proposal for a novel species within the genus *Fontibacillus*, for which the name *Fontibacillus panacisegetis* sp. nov. is proposed.

Emended description of the genus *Fontibacillus* Saha *et al.* 2010

The description is as given by Saha *et al.* (2010) with the following amendments. Cells are mesophilic, catalase-

Table 2. Cellular fatty acid composition (%) of strain P11-6^T and the type strains of related taxa

Strains: 1, *Fontibacillus panacisegetis* sp. nov. P11-6^T; 2, *Fontibacillus aquaticus* DSM 17643^T; 3, *Paenibacillus anaericanus* DSM 15890^T; 4, *Paenibacillus ginsengisoli* KCTC 13931^T. Fatty acids are listed using standard abbreviations (number of carbon atoms: number of double bonds). ND, Not detected.

Fatty acid	1	2	3	4
Saturated straight-chain				
C _{14:0}	3.6	6.1	5.7	5.5
C _{15:0}	6.5	3.2	1.2	4.7
C _{16:0}	5.7	11.4	9.1	8.2
Saturated iso-branched				
C _{14:0}	1.8	1.7	4.0	3.8
C _{15:0}	4.3	6.9	5.5	4.7
C _{16:0}	3.2	4.7	8.3	4.0
C _{17:0}	0.8	2.6	1.0	0.5
Saturated anteiso-branched				
C _{15:0}	64.0	53.3	60.7	64.0
C _{17:0}	4.8	7.0	3.1	1.4
Unsaturated				
C _{16:1ω11c}	2.4	ND	0.3	0.8
Summed feature 5*	2.9	3.2	1.0	2.5

*Summed feature 5 comprises iso-C_{17:1} I and/or anteiso-C_{17:1} B, which could not be separated by the MIDI system.

negative, oxidase-positive, Gram-stain-negative, motile rods. No growth is observed in the presence of 3 % NaCl. The major fatty acid is anteiso-C_{15:0}. Predominant polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethandamine (PE), APGL and PN2. The DNA G + C content ranges from 41.9 to 45.8 mol%.

Description of *Fontibacillus panacisegetis* sp. nov.

Fontibacillus panacisegetis (pa.na.ci.se.ge'tis. N.L. n. *Panax-acis* scientific name of ginseng; L. gen. n. *segetis* of the soil; N.L. gen. n. *panacisegetis* of soil of a ginseng field).

Cells are Gram-stain-negative, facultatively anaerobic rods (0.5–0.8 × 2.5–6.0 µm). Motile by means of peritrichous flagella. Terminal to subterminal, ellipsoidal spores are formed in swollen sporangia. Forms semi-translucent, circular, white colonies on TSA. Oxidase-positive but catalase-negative. Growth occurs at 15–42 °C (optimum 25 °C) and pH 6.0–8.5 (optimum pH 7.0). Growth occurs in the presence of up to 2 % NaCl, but not with 3 % NaCl. Anaerobic growth is observed, regardless of the presence of nitrate. Indole and H₂S are not produced. Nitrate is reduced but nitrite is not. Negative for citrate utilization. The methyl red test is negative, but the Voges–Proskauer test is positive. Aesculin, casein (weakly), DNA, *o*-nitrophenyl-β-D-galactopyranoside and Tween 20 (weakly) are hydrolysed, but gelatin, starch, Tween 80 and tyrosine are not. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase and urease are not produced. On egg yolk agar, lecithinase activity is absent. In the API 50CH gallery, acid is produced from *N*-acetylglucosamine, aesculin, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, glycerol, glycogen, inulin, lactose, maltose, D-mannose, melibiose, methyl α-D-glucoside, methyl β-D-xyloside, raffinose, D-salicin, starch, sucrose, turanose and D-xylose, but not from D-adonitol, D-arabinose, D- or L-arabitol, dulcitol, erythritol, D- or L-fucose, gluconate, inositol, 2- or 5-ketogluconate, D-lyxose, D-mannitol, melezitose, methyl α-D-mannoside, L-rhamnose, D-ribose, D-sorbitol, L-sorbose, D-tagatose, trehalose, xylitol or L-xylose. L-Arabinose, cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, glycerol, glycogen, lactose, maltose, D-mannose, melibiose, D-salicin, starch, sucrose and D-xylose are utilized as sole sources of carbon and energy, but acetate, *N*-acetylglucosamine, L-alanine, amygdalin, L-arabitol, citrate, L-fucose, gluconate, L-histidine, *myo*-inositol, DL-lactate, malate, malonate, D-mannitol, melezitose, L-proline, propionate, raffinose, L-rhamnose, D-ribose, L-serine, D-sorbitol, trehalose and turanose are not. According to API ZYM test results, leucine arylamidase, α-chymotrypsin, α-galactosidase and β-galactosidase are present, but alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-

mannosidase and α-fucosidase are absent. Susceptible to amikacin, ceftazidime, ceftriaxone, cephalothin, ciprofloxacin, colistin, doxycycline, gentamicin, imipenem, novobiocin, polymyxin B and tobramycin. Resistant to bacitracin, norfloxacin and oxacillin. Whole-cell hydrolysates contain *meso*-diaminopimelic acid and glucose. The only isoprenoid quinone is MK-7. The fatty acid anteiso-C_{15:0} is predominant. The major polar lipids are DPG, PG, PE, APGL, PN2 and UL2.

The type strain is P11-6^T (=KCTC 13564^T =CECT 7605^T), isolated from soil of a ginseng field. The DNA G + C content of the type strain is 41.9 mol%.

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