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Bacillus cheonanensis sp. nov. Isolated from Near Poultry Farm Soil[§]

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A novel bacterial strain, designated PFS-5^T, was isolated from the soil environment with feces of a live poultry farm located in Cheonan, Republic of Korea. Strain PFS-5^T was Gram-staining-positive, motile, strictly aerobic bacterium, rod-shaped, and endospore-forming. The strain contained *meso*-diaminopimelic acid in their peptidoglycan and MK-7 menaquinone. The major fatty acids were anteiso-$C_{15:0}$ (44.2%), $C_{16:0}$ (22.2%), and iso-$C_{15:0}$ (16.7%). The DNA G+C content was 40.1 mol%. Comparative 16S rRNA gene sequence analysis identified strain PFS-5^T in the genus *Bacillus*, exhibiting the highest level of sequence similarity with type strain of *B. herbersteinensis* D-1,5a^T (96.9%), *B. humi* LMG 22167^T (96.7%), *B. alkalitelluris* BA288^T (96.1%), *B. litoralis* SW-211^T (96.0%), and *B. luteolus* YIM 93174^T (95.5%). The major polar lipids of PFS-5^T were diphosphatidylglycerol and phosphatidylglycerol. On the basis of result from poly-phasic data, strain PFS-5^T represents a novel species, for which the name *Bacillus cheonanensis* sp. nov. is proposed (Type strain PFS-5^T = KACC 17469^T = JCM 19333^T).

Keywords: poultry farm soil, *Bacillus*, polar lipid

Introduction

The genus *Bacillus* (family *Bacillaceae*, phylum *Firmicutes*) was first described by Cohn in 1872. The number of Bacillus species has increased considerably since then. It is one of the largest bacterial genera, including 276 species and 7 subspecies (http://www.bacterio.net/; Euzéby, 2013). *Bacillus* species obligate aerobes or facultative anaerobes, spore-forming, halophilic or halotolerant, Gram-positive rods, and positive for catalase (Turnbull, 1996). Members of the genus *Bacillus* have been isolated from various foods and environments that include a tidal flat, dust, sediments, jeotgal, ginseng root, sandy soil, and brown alga (Venkateswaran *et al.*, 2003; Ivanova *et al.*, 2004; Bae *et al.*, 2005; Heyrman *et al.*, 2005b; Yoon and Oh, 2005; Lee *et al.*, 2008; Qiu *et al.*, 2009). During a study of bacterial populations associated with fowl manure, one isolate PFS-5^T, was isolated and subjected to polyphasic investigations to determine its taxonomic position.

Materials and Methods

Bacterial strain

Soil samples were collected from near a poultry farm in Cheonan city, Republic of Korea (36°43′01″N, 128°05′66″E), using the standard dilution plating technique on nutrient agar (NA; BD, USA) with 3% NaCl and 0.05% yeast extract at 30°C for 4 days. After primary isolation and purification, strain PFS-5^T was cultivated at 30°C on the same medium. The isolate was routinely cultured on this medium and preserved as a glycerol suspension (20% of glycerol and 80% of distilled deionized H2O, v/v) at -80°C. Reference strains, *B. humi* KACC 11370^T, *B. litoralis* KACC 12148^T, *B. herbersteinensis* KACC 12171^T, *B. luteolus* KCTC 13210^T, and *B. alkalitelluris* KCTC 3947^T were obtained from the Korean Agricultural Culture Collection (KACC) and Korean Collection for Type Culture (KCTC), respectively.

Phylogenetic analysis

Bacterial genomic DNA was extracted using an InstaGene^{TM} Matrix kit (Bio-Rad, USA). 16S rRNA gene was amplified using universal primers (Yoon *et al.*, 1998) using the GeneAmp PCR system 9700 (Applied Biosystems, USA). The 16S rRNA gene of strain PFS-5^T was sequenced using 27F, 518F, 800R, and 1492R primers (Lane, 1991). The identification of phylogenetic neighbors was performed using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The determined sequences (1,429 bp) were manually confirmed and aligned with reference 16S rRNA gene sequences available in GenBank and multiple alignments were computed using BioEdit (Hall, 1999). Phylogenetic trees were constructed (Fig. 1) according to the maximum likelihood (ML), neighbor-joining (NJ; Kimura-2-parameter distance model), maximum-parsimony (MP) and Bayesian inference (BI) using the Molecular Evolutionary Genetics Analysis (MEGA) version 5.10 (Tamura *et al.*, 2011). To evaluate the phylogenetic trees, bootstrap analysis with 1,000 sample replications was performed.

Phenotypic characteristic

Cells of strain PFS-5^T and references grown on modified NA agar at 30°C for 3 days were used for physiological and biochemical tests and the results were recorded for 5 days. To observe cell morphology, cells grown on modified NA for 3

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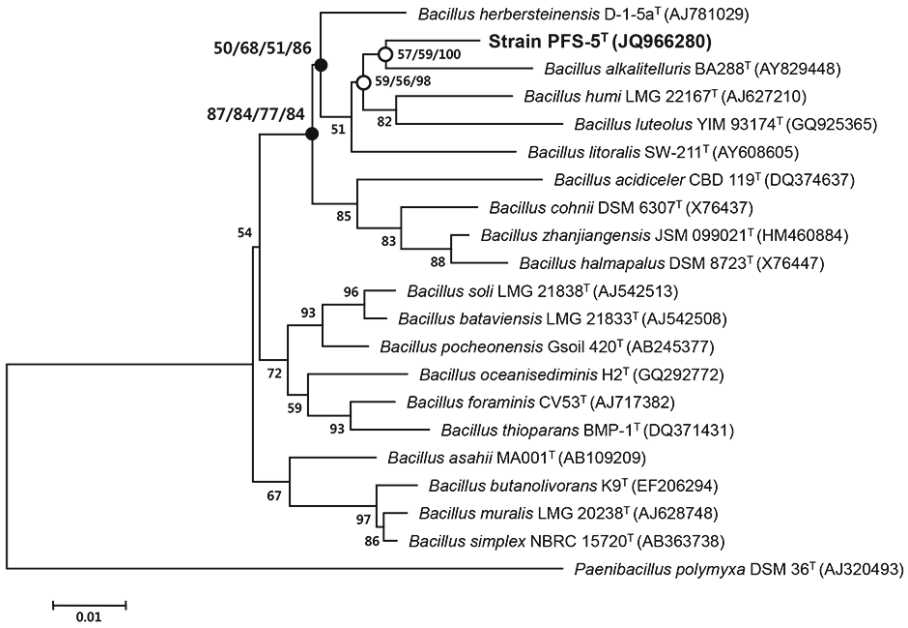

Fig. 1. Maximum likelihood tree showing the phylogenetic position of novel species based on 16S rRNA gene sequences. Evolutionary distances were computed using the Tamura-Nei model. Closed circle represents that the corresponding branches were recovered in the neighbor-joining (NJ), maximum-parsimony (MP) and Bayesian inference trees. Open circle means that the corresponding branches were recovered in the NJ which is Kimura 2-parameter distance model (Kimura, 1980) and Bayesian inference trees. Bootstrap values (>50%) based on 1000 replications are shown. *Paenibacillus polymyxa* DSM 36^T (AJ320493) was used as an out-group. Bar, 0.01 nucleotide substitutions per nucleotide position.

days at 30°C were analyzed by using CM 20 transmission electron microscope (TEM; Philips, Netherlands). The flagellum type was examined by TEM using cells from the exponential growth phase. Cells were mounted on Formvar-coated copper grids and negatively stained with 1% potassium phosphotungstate (pH 7.0) prior to examination. Gram-reaction was performed using a commercial Gram staining kit (Sigma-Aldrich, USA). Motility test was carried out on modified NA medium containing 0.5% of agar through the hanging-drop method (Skerman, 1967) using a CHT light microscope (Olympus, Japan) at ×1000 magnification. Endospores were stained using malachite green staining (Smibert and Krieg, 1994). Growth at 4, 10, 15, 20, 25, 30, 37, 40, and 45°C was examined in nutrient broth. Growth at pH 5.0–11.0 (at intervals of 0.5 pH units) was tested in modified nutrient broth adjusted with 0.1 M citrate buffer (pH 5.0), 0.2 M sodium phosphate buffer (pH 6.0–8.0), 0.2 M glycine-NaOH buffer (pH 9.0–10.0) and 0.2 M sodium carbonate buffer (pH 11.0) as described by Gomori (1995). The influence of salinity was tested on modified NA medium supplemented in 1% increments with 0–15% w/v NaCl. Anaerobic growth determined after incubation for 15 days on modified NA medium in a jar with the GasPak anaerobic system (BBL, USA). Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and oxidase reagents (bioMérieux, France), respectively. Methyl red and Voges-Proskauer tests were performed as previously described (Smibert and Krieg, 1981). Hydrolysis of Tween 20, Tween 40, Tween 60, Tween 80, casein, and starch were determined using the methods of Smibert and Krieg (1994). API 20E and API 50CHB test strips (bioMérieux, France) were used to analyze biochemical and physiological characteristic and sugar fermentation patterns. The API 50CHB strips were read after 3 days at 30°C. Enzyme activity tests were conducted by using the API ZYM galleries, according to the manufacturer's instructions (bioMérieux). The API 20E was read after 24 and 48 h of incubation at 30°C and the API ZYM galleries were read after 4 h of incubation at 37°C.

DNA G+C content and Chemotaxonomy

The genomic DNA from strains was extracted and purified as described by Moore and Dowhan (1995). The DNA G+C content was determined as described by Mesbah *et al.* (1989) using reversed-phase high-performance liquid chromatography (HPLC, Supelco). The cell-wall peptidoglycan structure was determined by using the TLC method as described by Staneck and Roberts (1974). Fatty acid methyl esters were obtained from fresh wet biomass, grown on modified NA at 30°C for three days, by saponification, methylation, and extraction as described by Aslam *et al.* (2009), and the fatty acids were separated, identified, and quantified according to the protocol of the Sherlock Microbial Identification System (MIDI) version 6.0 and the RTSBA6. The gas chromatograph was Agilent 87 Technologies 7890A GC system. Isoprenoid quinone was analyzed by HPLC as describe by Groth *et al.* (1996). Polar lipids were analyzed according to Minnikin *et al.* (1984).

Results and Discussion

Phylogenetic analysis

In the phylogenetic trees reconstructed using the various algorithms, strain PFS-5^T belongs to the genus *Bacillus*, clustering with *B. herbersteinensis* D-1-5a^T, *B. humi* LMG 22167^T, *B. alkalitelluris* BA288^T, *B. litoralis* SW-211^T, and *B. luteolus* YIM 93174^T supported by 63% average bootstrap value (Fig. 1). Strain PFS-5^T showed levels of 16S rRNA gene sequence similarity of 95.5–96.9% to the most closely related clade, with the highest similarities to *B. herbersteinensis* D-1-5a^T (96.9%), *B. humi* LMG 22167^T (96.7%), *B. alkalitelluris* BA288^T (96.1%), *B. litoralis* SW-211^T (96.0%), and *B. luteolus* YIM 93174^T (95.5%).

Phenotypic characteristic

All strains were used as reference strains for most phenotypic tests. After incubation for 3 days on modified NA medium, colonies of strain PFS-5^T were creamy-white, opaque, irregular, and rod shape (approximately 0.6–0.8 μm wide, 2.8–3.2 μm long) (Supplementary data Fig. S1). Strain PFS-5^T was Gram-staining-positive, strictly aerobic, motile, catalase-positive, oxidase-negative, and spore forming. Our isolate grew at 15–40°C (optimum 30°C), at pH 6.0–9.0 (optimum pH 7.0), and in 0–8% NaCl (optimum 3.0%, w/v). Colony pigmentation and cell size of PFS-5^T are also distinct from the reference strains. Based on the hydrolysis experiments of casein, starch, aesculin, gelatin, urea, and Tween 60, strain PFS-5^T exhibited dissimilar hydrolysis ability compared to that of the reference strains. Especially for the hydrolysis of Tween 80, strain PFS-5^T exclusively exhibited a hydrolysis whereas all of the reference strains were inactive. The isolate also exhibited differences in acid production from nine substances including N-acetylglucosamine compared to that of the reference strains (Table 1). Particularly in utilizing glucose, strain PFS-5^T exclusive was active when all other

Table 1. Comparison of the characteristics of strain PFS-5 with type species of closest phylogenetic neighbors
Strains: 1, PFS-5^T; 2, *B. luteolus* KCTC 13210^T; 3, *B. alkalitelluris* KCTC 3947^T; 4, *B. litoralis* KACC 12148^T; 5, *B. humi* KACC 11370^T; 6, *B. hebersteinensis* KACC 12171^T. CW, creamy white; PT, pale tangerine; YW, yellowish white; W, white; C, Cream; +, positive; -, negative; w, weak positive.

Characteristic	1	2	3	4	5	6
Colony pigmentation	CW	PT	CW	YW	W	C
Cell length (μm)	2.8–3.2	2.2–2.5	2.2–2.3	2.2–3.6	2.2–4.4	2.2–4.4
Nitrate reduction	-	-	-	-	+	-
Anaerobic growth	-	-	+	-	w	-
Range of (optimum)						
Temperature (°C)	20–37 (30)	15–45 (30)	15–40 (30)	4–45 (37)	20–40 (30)	4–28 (25)
pH	6–9 (7)	6–8 (7)	7–11 (9)	5–9 (7)	6–9 (7)	7–11 (8)
NaCl tolerance (%, w/v)	0–8 (3)	0–10 (0–2)	0–4 (0-1)	0–10 (2-3)	4–9 (7)	0–5 (1)
Catalase activity	+	+	-	+	+	+
Oxidase activity	-	-	+	-	+	+
Hydrolysis of:						
Casein	-	-	-	+	-	-
Starch	-	-	+	+	-	+
Aesculin	+	-	+	+	+	-
Gelatin	-	-	-	+	-	-
Urea	-	+	-	-	+	-
Tween 60	+	+	-	+	-	+
Tween 80	+	-	-	-	-	-
Acid production from:						
N-Acetylgulucosamine	-	-	-	+	-	-
Amygdalin	-	+	-	+	-	-
L-Arabinose	-	-	+	+	-	+
Cellobiose	-	-	+	-	-	-
D-Fructose	-	+	-	+	-	-
Galactose	-	-	-	+	-	+
Glucose	+	-	-	+	-	-
Raffinose	+	-	-	+	-	-
Salicin	+	-	+	+	-	-
Enzymic activities						
Acid phosphatase	+	-	-	+	+	-
α-Chymotrypsin	-	-	+	+	+	+
Cystine arylamidase	+	-	+	-	+	-
β-Galactosidase	+	-	+	-	-	-
α-Gucosidase	+	+	-	-	-	-
β-Gucosidase	+	-	+	+	-	-
β-Glucuronidase	+	-	+	-	+	-
Leucine arylamidase	+	+	+	-	+	-
Lipase	-	+	-	-	-	+
Trypsin	-	-	+	+	+	-
Valine arylamidase	+	-	+	-	+	-
DNA G+C content (mol%)	40.1	36.9[a]	37.9[b]	35.2[c]	37.5[d]	36.6[e]

[a] Data from Shi *et al.* (2011). [b] Data from Lee *et al.* (2008). [c] Data from Yoon *et al.* (2005). [d] Data from Heyrman *et al.* (2005a). [e] Data from Wieser *et al.* (2005).

Table 2. Fatty acid content of strain PFS-5^T and its closest neighbors
Strains: 1, PFS-5^T; 2, *B. luteolus* KCTC 13210^T; 3, *B. alkalitelluris* KCTC 3947^T; 4, *B. litoralis* KACC 12148^T; 5, *B. humi* KACC 11370^T; 6, *B. hebersteinensis* KACC 12171^T. Values are percentages of total fatty acids; -, not detected. Data were obtained in this study.

Fatty acid	1	2	3	4	5	6
$C_{14:0}$	4.5	0.4	0.8	-	0.5	-
$C_{16:0}$	22.2	1.1	4.6	2.6	0.9	2.1
iso-$C_{14:0}$	8.6	9.6	8.3	11.7	11.7	11.2
iso-$C_{15:0}$	16.7	33.8	18.1	31.9	18.9	29.5
iso-$C_{16:0}$	3.9	9.6	4.1	4.2	5.0	6.4
anteiso-$C_{15:0}$	44.2	20.2	39.7	32.8	52.1	20.6

strains were silent. Dissimilarities between PFS-5^T and the reference strains were augmented in the enzyme activity experiments. Enzyme activities of PFS-5^T for 11 different enzymes including acid phosphatase were different from those of the reference strains, suggesting that the isolate is a new strain. The phenotypic characteristics, strongly suggesting that PFS-5^T is a novel bacterium (Table 1).

DNA G+C content and Chemotaxonomy

DNA G+C content of the strain PFS-5^T was 40.1 mol%. The cell-wall peptidoglycan of the strain was A1γ type, containing meso-diaminopimelic acid as the diagnostic diamino acid (as is the case in the great majority of the members of the genus *Bacillus*; Claus and Berkeley, 1986). The fatty acids of strain PFS-5^T were anteiso-$C_{15:0}$ (44.2%), $C_{16:0}$ (22.2%), iso-$C_{15:0}$ (16.7%), iso-$C_{14:0}$ (8.6%), $C_{14:0}$ (4.5%), and iso-$C_{16:0}$ (3.9%). The proportion of $C_{14:0}$ and $C_{16:0}$ were larger than those recorded for other type species and smaller than iso-$C_{14:0}$, iso-$C_{15:0}$, and iso-$C_{16:0}$ (Table 2). The fatty acid profiles of strain PFS-5^T contained the major compounds $C_{14:0}$, iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, and iso-$C_{16:0}$, which are features of numerous taxa within the bacilli. The presence of branched, saturated fatty acids as the major fatty acids is consistent with the profiles for the type strains of the genus *Bacillus* (Kämpfer, 1994). The isoprenoid quionone was MK-7. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, unknown phospholipid and unknown aminophospholipid (Supplementary data Fig. S2).

Taxonomic conclusion

Based on the genetic, phenotypic, and chemotaxonomic features and phylogenetic differences between the novel strain and previously described species within the genus *Bacillus*, strain PFS-5^T represents a novel species, for which the name *Bacillus cheonanensis* sp. nov. is proposed.

Description of *Bacillus cheonanensis* sp. nov.

Bacillus cheonanensis (che.on.an.en'sis N.L. masc. adj. cheonanensis pertaining to Cheonan, Republic of Korea, the geographical origin of the type strain).

Cells are Gram-staining-positive, motile, strictly aerobic, spore-forming, and rod-shaped (approximately 0.9–1.0 μm wide, 2.8–3.2 μm long). Colonies are opaque, irregular, and creamy-white on NA medium at 30°C for 3 days. Growth occurs at 15–40°C (optimum 30°C), at pH 6.0–9.0 (optimum pH 7.0), and presence of 0–9% (w/v) NaCl (optimum 3%). Spherical endospores are produced in a terminal position. Methly red test is positive but Vogs-Proskauer test is negative. Indole and H_2S are not produced. In the API 50CHB system, strain PFS-5^T is positive for glucose, raffinose, salicin but negative for glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl-β-D-xylopyranside, galactose, fructose, mannose, sorbose, dulcitol, inositol, mannitol, sorbitol, methyl-α-D-mannopyranside, methyl-α-D-glucoside, *N*-acetyl-glucosamine, amygdalin, arbutin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, and 5-keto-gluconate. On API 20E, there is positive results for β-galactosidase but negative reactions for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, utilization of citrate, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, and production of H_2S. In the API ZYM system is positive for acid phosphatase, crysteine arylamidase, β-galctosidase, α-glucosidase, β-glucosidase, β-glucuronidase, leucine arylamidase, and valine arylamidase, but negative for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), α-chymotrypsin, trypsin, naphtol-AS-BI-phosphohydrolase, α-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The strain PFS-5^T hydrolyses aesculin, Tween 60, and Tween 80, but does not hydrolyze casein, gelatin, starch, urea, Tween 20, Tween 40, and dextrin. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid (A1γ type). The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, unknown phospholipid and unknown aminophospholipid. The predominant menaquinone is MK-7 and DNA G+C content of the type strain is 40.1 mol%. The major fatty acids of the strain PFS-5^T were anteiso-$C_{15:0}$, $C_{16:0}$, and iso-$C_{15:0}$. The type strain, PFS-5^T (=KACC 17469^T =JCM 19333^T), was isolated from soil of near live poultry farm at Cheonan, Republic of Korea.

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