Bacillus fengqiuensis sp. nov., isolated from a typical sandy loam soil under long-term fertilization

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A Gram-staining-positive, endospore-forming, moderately alkalophilic bacterium, strain NPK15T, was isolated from a typical sandy loam soil under long-term NPK fertilization in northern China and was subjected to a polyphasic taxonomic study. The diamino acid of the cell-wall peptidoglycan of strain NPK15T was found to be meso-diaminopimelic acid and the cell-wall sugars were xylose, glucose and traces of mannose. The only respiratory quinone found in strain NPK15T was menaquinone 7 (MK-7). The major cellular fatty acids were iso-C15:0, anteiso-C15:0, C16:0 and C16:1ω6c/C16:1ω7c. The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. Phylogenetic analysis of the strain based on its 16S rRNA gene sequence showed that it was related most closely to ‘Bacillus thaonhiensis’ KACC 17216 (99.59 %), B. songkensis KCTC 13881T (99.52 %) and B. abyssalis CCTCC AB 2012074T (99.00 %). DNA–DNA hybridization results indicated that the strain was distinct from other species of the genus Bacillus, the degree of relatedness being 35.4 % with B. abyssalis CCTCC AB 2012074T, 39.7 % with B. songkensis KCTC 13881T and 51.2 % with ‘B. thaonhiensis’ KACC 17216. The DNA G+C content of strain NPK15T was 45.5 mol%. Phenotypic, chemotaxonomic and molecular analyses identified strain NPK15T as a member of a novel species of the genus Bacillus, for which the name Bacillus fengqiuensis sp. nov. is proposed. The type strain is NPK15T (=DSM 26745T=CCTCC AB 2013156T).

The genus Bacillus was established to include three species of rod-shaped bacteria, Bacillus subtilis, B. anthracis and ‘B. ulna’ (Cohn, 1872). At the time of writing, the genus Bacillus, in the phylum Firmicutes, consisted of 281 species with validly published names (http://www.bacterio.net/bacillus.html), with B. subtilis as the type species. Members of the genus are able to form endospores that resist many adverse conditions and have been isolated from a wide variety of environments, including soils (Chen et al., 2011b; Jung et al., 2009; Madhaiyan et al., 2010), fresh and marine water (Baik et al., 2010; Yoon et al., 2004), volcanic ash (Logan et al., 2004), deep-sea sediment (You et al., 2013; Zhang et al., 2010), hypersaline sites (Bagheri et al., 2012; Hong et al., 2012) and inner tissues of plants and animals (Bibi et al., 2011; Chen et al., 2011a). In this paper, during an investigation of the diversity of the bacterial population of a typical sandy loam soil under long-term NPK fertilization, an endospore-forming, Gram-stain-positive bacterium, strain NPK15T, was isolated and found to have morphological properties consistent with those of members of the genus Bacillus.

Strain NPK15T was isolated from a typical sandy loam soil which was part of the State Experimental Station for Agro-Ecology, Fengqiu county, Henan province, China (35° 00’ N 114° 24’ E). The soil was a typical soil in the northern China region, with a profile of sandy loam (about 9 % clay, 21.8 % slit) in the plough layer and loam in the subsoil, and was derived from alluvial sediments of the Yellow River and classified as an aquic incertisol (a calcareous fluvo-aquic soil). A standard dilution-plating technique was used to isolate the strain on nutrient agar (5 g peptone, 3 g meat extract, 1 l distilled water; pH 7.2–7.4) at 30 °C for 2 days. Strain NPK15T was maintained on nutrient agar at 4 °C and stored as 20 % (v/v) glycerol suspensions at −20 °C.

To characterize strain NPK15T phenotypically, standard phenotypic tests were performed according to general protocols (Gordon et al., 1974; Lányi, 1987). Cellular morphology and motility were examined by light microscopy (CX21; Olympus) and transmission electron microscopy.

Abbreviation: Dpm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NPK15T is KC291653.

Five supplementary figures and a supplementary table are available with the online version of this paper.
were hydrolysed (0.5 M H2SO4, 100 °C; Shimadzu). For analysis of the cell-wall sugars, cells amino acids was carried out by gas chromatography (GC (bioMérieux) according to the manufacturer’s instructions. The oxidase reaction was performed on filter paper moistened with a 1% (v/v) aqueous solution of N,N,N',N'-tetramethyl p-phenylenediamine. Anaerobic growth was studied for 7 days at 30 °C on anaerobic agar (Claus & Berkeley, 1986). Hydrolysis of Tween 80 was determined as described by Cowan & Steel (1965). Susceptibility to antibiotics was tested on nutrient agar plates using discs containing the following amounts of antibiotics: ampicillin, 10 μg; rifampicin, 5 μg; streptomycin, 10 μg; kanamycin, 30 μg; gentamicin, 10 μg; tetracycline, 30 μg; chloramphenicol, 5 μg; erythromycin, 15 μg; polymixin B, 300 U. Metabolic properties and enzyme activities were tested using API 20 E, API 20 NE, API 50 CHB/E and API ZYM systems (bioMérieux) according to the manufacturer’s instructions. Elucidation of the peptidoglycan structure was carried out as described by Schumann (2011). Quantitative analysis of amino acids was carried out by gas chromatography (GC 14A; Shimadzu). For analysis of the cell-wall sugars, cells were hydrolysed (0.5 M H2SO4, 100 °C, 2 h) and the sugars were analysed by TLC on cellulose plates according to Stanek & Roberts (1974). For polar lipids and respiratory quinone analyses, cell mass of strain NP1K15T was harvested from nutrient agar after incubation for 6–7 h at 37 °C. Polar lipids were extracted from 100 mg freeze-dried cell material and separated by two-dimensional silica-gel TLC (art. no. 818 135; Macherey-Nagel) (Tindall et al., 2007). For analysis of cellular fatty acids, biomass of strain NP1K15T was harvested from nutrient agar after incubation for 24 h at 28 °C (exponential stage) according to the MIDI handbook. Fatty acid methyl esters from 40 mg cells scraped from Petri dishes were obtained by saponification, methylation and extraction using minor modifications of the method of Miller (1982) and Kuykendall et al., (1988) and were separated using the Sherlock Microbial Identification System (TSBA6, version 6.1; MIDI, Microbial ID), which consisted of an Agilent model 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm × 25 m), a flame-ionization detector, Agilent model 7683A automatic sampler and an HP computer with MIDI database (Hewlett Packard). Respiratory quinones were extracted from 100 mg freeze-dried cells and were separated into their different classes (menaquinones, ubiquinones, etc.) by TLC on silica gel (art. no. 805 023; Macherey-Nagel). UV-absorbing bands corresponding to the different quinone classes were removed from the plate and analysed further by HPLC (LDC Analytical; Thermo) fitted with a reversed-phase column (2 mm × 125 mm, 3 μm, RP18; Macherey-Nagel). Respiratory quinones were detected at 269 nm. Analyses of peptidoglycan structure, cell-wall sugars, polar lipids and respiratory quinones were carried out by the Identification Service of the DSMZ, Braunschweig, Germany.

For analysis of the 16S rRNA gene sequence, bacterial DNA was extracted using a Bacterial DNA kit (D3350; Omega) according to the manufacturer’s instructions. The 16S rRNA gene was amplified by PCR using universal primers according to the methods of Timke et al. (2005) and the amplification product was sequenced directly automatically using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer. Multiple alignments of data and phylogenetic analysis were performed using the MEGA software (version 5.0) (Tamura et al., 2011). Distances were calculated according to the distance options with Kimura’s two-parameter model and clustering with the neighbour-joining, minimum-evolution and maximum-likelihood algorithms. Bootstrap values were determined based on 1000 replications. The neighbour-joining tree is shown in Fig. 1. The minimum-evolution and maximum-likelihood trees are available as Figs S1 and S2, available in the online Supplementary Material. The DNA G+C content of strain NP1K15T was determined as described by Mesbah et al. (1989) using reversed-phase HPLC. DNA–DNA hybridization was carried out as described by De Ley et al. (1970) using a UV/Vis spectrophotometer (UV1201; Rayleigh).

Strain NP1K15T formed off-white, circular to slightly irregular colonies with fimbriate edges that were about 0.5–2 mm in diameter after 2 days of incubation on nutrient agar at 30 °C. Cells of strain NP1K15T were Gram-staining-positive, motile rods (1.2–1.9 μm wide and 3.5–4.8 μm long) that occurred singly or in pairs (Fig. S3). Central or subterminal ellipsoidal endospores were observed in swollen sporangia (Fig. S4). The temperature range for growth was 20–45 °C, with optimum growth at 37 °C. The pH range for growth was pH 7.0–11.0, with optimum growth at pH 8.5. NaCl was tolerated at 0–2% (w/v). The results of the other physiological and biochemical analyses are summarised in Table 1 and the species description. Comparison of the closest relatives of strain NP1K15T showed that B. abyssalis CCTCC AB 2012074T and ‘B. thaonhiiensis’ KACC 17216 could grow slightly at 60 °C on marine agar 2216 and R2A medium, but that strain NP1K15T and B. songkensis KCTC 13881T could not grow at such high temperatures. The pH range for growth also differentiated strain NP1K15T from its closest relatives: strain NP1K15T could grow at pH 11.0, while the other three strains did not tolerate such extreme
pH levels. Other physiological and biochemical characteristics also supported the distinctiveness of strain NPK15\textsuperscript{T} from its close relatives (Table 1).

One- and two-dimensional TLC of the total hydrolysate of peptidoglycan (4 M HCl, 16 h, 100 °C) revealed the presence of the amino acids meso-diaminopimelic acid (Dpm), alanine and glutamic acid. The absence of amino acids like leucine, isoleucine and phenylalanine that occur in proteins but have never been detected in peptidoglycan indicated that the resulting peptidoglycan preparation was free of contaminating proteins. After derivatization to N-heptafluorobutyryl amino acid isobutyl esters, the molar amino acid ratio was determined by gas chromatography (GC 14A; Shimadzu) as 1.0 Ala : 0.9 meso-Dpm : 1.0 Glu. In addition, the peptides L-Ala–D-Glu and meso-Dpm–D-Ala could be detected by two-dimensional TLC after hydrolysis under milder conditions (4 M HCl, 45 min, 100 °C). From these data, the occurrence of the meso-Dpm peptidoglycan type in strain NPK15\textsuperscript{T} was concluded. The only respiratory
Table 1. Comparison of properties of strain NPK15<sup>T</sup> with those of the most closely related members of the genus *Bacillus*.

Strains: 1, NPK15<sup>T</sup>; 2, *B. abyssalis* CCTCC AB 2012074<sup>T</sup>; 3, *B. songklensis* KCTC 13881<sup>T</sup>; 4, *B. thaonhensis* KACC 17216. Data were taken from this study except the DNA G+C contents. All strains are positive for Gram-staining, catalase, citrate utilization, gelatinase, alkaline phosphatase, esterase (C4) and esterase lipase (C8). All strains are susceptible to ampicillin (10 μg), rifampicin (5 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), tetracycline (30 μg), chloramphenicol (5 μg), erythromycin (15 μg) and polymyxin B (300 U). All strains are negative for hydrolysis of Tween 80, production of indole and H<sub>2</sub>S, activities of β-galactosidase, arginine dihydrolase, urease, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, lipase (C14), cystine arylamidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, z-mannosidase and α-fucosidase and utilization of capric acid, trisodium citrate, L-xylose, methyl α-D-mannopyranoside, L-sorbose, L-rhamnose, dulcitol, glyogen, cellobiose, xylitol, D-tagatose and D-arabitol. +, Positive; w, weakly positive; −, negative.

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<td>Salicin</td>
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The peptidoglycan and quinone of strain NPK15\(^T\) are in agreement with those of numerous species of the genus *Bacillus*, including the type species, *B. subtilis* (Claus & Berkeley, 1986). These characteristics were significantly different from those of the closely related genera *Viridibacillus* (Albert et al., 2007), *Lysinibacillus* (Ahmed et al., 2007) and *Rummeliibacillus* (Vaishampayan et al., 2009).

The major fatty acid compounds (>10%) of strain NPK15\(^T\) were C\(_{16:1}\)\(\alpha\)/C\(_{16:1}\)\(\omega7c\) (31.1%), iso-C\(_{15:0}\) (22.6%), C\(_{16:0}\) (17.5%) and anteiso-C\(_{15:0}\) (11.5%). The presence of the fatty acids iso-C\(_{15:0}\), iso-C\(_{16:0}\) and anteiso-C\(_{15:0}\), which are characteristic of numerous taxa within the bacilli (Kämpfer, 1994), as major compounds supports the allocation of strain NPK15\(^T\) to the genus *Bacillus*. The relative larger percentage of saturated and unsaturated fatty acids could distinguish strain NPK15\(^T\) clearly from its phylogenetically closest relatives (Table S1). The polar lipids of strain NPK15\(^T\) were diphasatidylethanolamine, diphasatidylglycerol and phosphatidyglycerol in strain NPK15\(^T\) has also been reported for *B. subtilis* (Minnikin & Goodfellow, 1981). On the other hand, strain NPK15\(^T\) differs from *B. songkensis* KCTC 13881\(^T\) by the absence of two unidentified phospholipids, four unidentified aminophospholipids, an unidentified aminolipid, two unidentified glycolipids and an unidentified polar lipid (Kang et al., 2013). However, an unknown phospholipid, an unknown aminophospholipid and an unknown polar lipid were also present in *B. thaonhiensis* KACC 17216 (Van Pham & Kim, 2014). TLC analysis of the cell-wall sugars of strain NPK15\(^T\) revealed the presence of xylose, glucose and traces of mannose.

An almost-complete 16S rRNA gene sequence (1474 bp) was obtained from strain NPK15\(^T\) and subjected to similarity searches by using the sequence matching tool of the NCBI BLAST program (http://www.ncbi.nlm.nih.gov), the Ribosomal Database Project (http://rdp.cme.msu.edu) and EzBioCloud (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). Comparative 16S rRNA gene sequence

### Table 1. cont.

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<td>w</td>
<td>w*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>w</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Naphthol-AS-Bi-phosphohydrolase</td>
<td>-</td>
<td>w*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>z-Galactosidase</td>
<td>-</td>
<td>w*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\beta$-Glucuronidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\alpha$-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tryptophan deaminase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>45.5</td>
<td>43.1*</td>
<td>41.4</td>
<td>40.7c</td>
</tr>
</tbody>
</table>

*Result from this study differs compared with the original description.
†Data from: a, You et al. (2013); b, Kang et al. (2013); c, Van Pham & Kim (2014).
analysis of strain NPK15T and related strains showed similarities of 99.00, 99.52 and 99.59 % to B. abyssalis CCTCC AB 2012074T, B. songkensis KCTC 13881T and ‘B. thaonhiensis’ KACC 17216, respectively. The phylogenetic tree based on 16S rRNA gene sequences of strain NPK15T and related species of the genus Bacillus is shown in Fig. 1. In the phylogenetic tree based on the neighbour-joining algorithm, strain NPK15T formed an independent cluster with B. abyssalis CCTCC AB 2012074T, B. songkensis KCTC 13881T and ‘B. thaonhiensis’ KACC 17216 with a bootstrap value of 99 %. Similar tree topologies were seen in the minimum-evolution and maximum-likelihood trees (Figs S1 and S2). The DNA G+C content of strain NPK15T was 45.5 mol%. DNA–DNA hybridization studies showed relatively low relatedness with B. abyssalis CCTCC AB 2012074T (35.4 ± 0.8 %), B. songkensis KCTC 13881T (39.7 ± 1.5 %) and ‘B. thaonhiensis’ KACC 17216 (51.2 ± 0.2 %). All of these values are significantly lower than 70 %, the threshold value recommended for the assignment of strains to different genomic species (Wayne et al., 1987).

In recent years, the use of the fatty acid composition in taxonomic studies has been increasingly emphasized; however, it is well known that their resolution is limited. The combination of 16S rRNA gene sequence data and phenotypic properties including physiology, morphology and biochemistry is still necessary for taxonomic arrangement (You et al., 2013). The significant differences in the cellular fatty acid profile and phenotypic properties can be used to distinguish strain NPK15T from B. abyssalis CCTCC AB 2012074T, B. songkensis KCTC 13881T and ‘B. thaonhiensis’ KACC 17216. Therefore, it is concluded that the isolate should be placed in the genus Bacillus. On the basis of the taxonomic data described above, strain NPK15T represents a novel species of the genus Bacillus, for which the name Bacillus fengqiuensis sp. nov. is proposed.

**Description of Bacillus fengqiuensis sp. nov.**

Bacillus fengqiuensis (feng.qiu.en’sis. N.L. masc. adj. fengqiuensis referring to Fengqiu city, Henan Province, PR China, where the type strain was isolated).

Cells are motile, rounded-ended rods (1.2–1.9 μm wide and 3.5–4.8 μm long) that occur singly or in pairs. Gram-stain-positive, but Gram-stain-variable in older cultures. Motile by means of a single lateral flagellum. Central or subterminal ellipsoidal endospores are observed in swollen sporangia. Colonies are matt, circular to slightly irregular with fimbriate edges, slightly raised, off-white and 2–4 mm in diameter after 2 days at 37 °C on nutrient agar. Colonies produce red pigments from the centre to the edge after 4–7 days on TSA under the same cultivation conditions. The temperature range for growth is 20–45 °C; optimum growth occurs at 37 °C. The pH range for growth is pH 7.0–11.0, with optimum growth at pH 8.5. NaCl is tolerated at 0–2 % (w/v). The preferred growth medium is nutrient agar. Aerobic, oxidase-negative and catalase-positive. Starch and Tween 80 are not hydrolysed. In the API 20E strip (bioMérieux) incubated at 30 °C, o-nitrophenyl β-galactoside is not hydrolysed; tests for tryptophan deaminase and the Voges–Proskauer reaction are positive. In the API 50 CH gallery (bioMérieux), hydrolysis of aesculin is negative. Acid without gas is produced from the following carbohydrates in the API 50 CH gallery using the CHB suspension medium (bioMérieux): glycerol, erythritol, D- and L-arabinose, D-ribose, D-xylitol, methyl β-D-xylpyranoside, D-glucose, D-fructose, myo-inositol, D-mannitol, N-acetylglucosamine, maltose, sucrose, trehalose, inulin, melizitose and raffinose; weak acid reactions are detected for D-galactose, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, lactose (bovine origin), turanose, D-lyxose, D- and L-fucose, L-arabinitol, potassium gluconate, 2-keto-D-glucuronate and 5-keto-D-glucuronate. Acid is not produced from the following carbohydrates: L-xylitol, D-adonitol, D-mannose, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α-D-mannpyranoside, aesculin ferric citrate, cellobiose, melibiose, starch, glycogen, xylitol, gentiobiose, D-tagatose and D-arakinol. Cells are sensitive to filter-paper discs containing the following antibiotics: ampicillin (10 μg), rifampicin, (5 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), tetracycline (30 μg), chloramphenicol (5 μg), erythromycin (15 μg) and polymixin B (300 U). In API ZYM assays, reactions of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and α-glucosidase are positive; reactions of acid phosphatase and α-chymotrypsin are weakly positive, but reactions of lipase (C14), valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-Bi-phosphohydrolase, α- and β-galactosidase, α-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. The cell wall contains meso-Dpm as the diagnostic diamino acid. The cell-wall sugars are xylose, glucose and traces of mannose. Predominant polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The major respiratory quinone is MK-7. Major cellular fatty acids are C<sub>16:0</sub>3OH, C<sub>16:1</sub>ω7c, iso-C<sub>15:0</sub>, C<sub>16:0</sub>, anteiso-C<sub>15:0</sub> and anteiso-C<sub>15:0</sub>.

The type strain, NPK15T (=DSM 26745T=CCTCC AB 2013156T), was isolated from a typical sandy loam soil under long-term NPK fertilization at the State Experimental Station for Agro-Ecology, Fengqiu county, Henan province, PR China (35°00’ N 114°24’ E). The DNA G+C content of the type strain is 45.5 mol%.

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