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TAXONOMIC DESCRIPTION

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Paenibacillus konkukensis sp. nov., isolated from animal feed

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Abstract

A Gram-stain-positive, oxidase- and catalase-positive, aerobic, rod-shaped bacterium, designated strain SK-3146^T, was isolated from animal feed. Phylogenetic analysis, based on 16S rRNA gene sequence comparisons, revealed that the strain formed a distinct lineage within the genus *Paenibacillus* that was closely related to *Paenibacillus yunnanensis* JCM 30953^T (98.6 %), *Paenibacillus vulneris* CCUG 53270^T (98.0 %) and *Paenibacillus chinjuensis* DSM 15045^T (96.9 %). Cells were non-motile, endospore-forming and formed milky colonies on NA and R2A agar media. Growth of strain SK-3146^T occurred at temperatures of 18–45 °C, at pH 6.0–9.5 and between 0.5–3.0 % NaCl (w/v). The major menaquinone was MK-7, with lesser amounts of MK-6 present. The cell wall peptidoglycan of strain SK-3146^T contained *meso*-diaminopimelic acid. The major fatty acids were anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The major polar lipids were diphosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content was 53.8 mol% and the DNA–DNA hybridization relatedness values between strain SK-3146^T and *P. yunnanensis* JCM 30953^T and *P. vulneris* CCUG 53270^T were 26.13±0.8 % and 38.7±0.6 %, respectively. The phenotypic, phylogenetic and chemotaxonomic results indicate that strain SK-3146^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus konkukensis* sp. nov. is proposed. The type strain is SK-3146^T (=KACC 18876^T=LMG 29568^T).

The genus *Paenibacillus* represents a group of *bacilli* first described by Ash *et al.* [1], with its description emended by Shida *et al.* [2]. Members of the genus are defined as Gram-stain-positive or variable, facultatively anaerobic or strictly aerobic, non-pigmented, rod-shaped, motile and endospore-forming bacteria [1–4]. The DNA G+C contents are in the range of 39–59 mol% [5]. Species of the genus are widespread, and hence have been isolated from a variety of sources, including rhizospheres of trees [6, 7], flowers [8], gamma-irradiated Antarctic soil [9], duckweed [10], nodules of *Lupinus albus* [11], tidal flats [12], water springs [13], rice seed [14], iron mineral soil [15] and necrotic wounds [16]. The cell wall peptidoglycan of members of the genus *Paenibacillus* is *meso*-diaminopimelic (meso-DAP) and menaquinone-7 (MK-7) is the predominant menaquinone. Diphosphatidylglycerol (DPG) is the major polar lipid in all the members of the genus *Paenibacillus* for which polar lipid data are available [5]. The predominant cellular fatty acid is anteiso-$C_{15:0}$ [17]. The species of this genus are very significant from an industrial and economical point of view [4], as many species have been shown to be important for agricultural, horticultural (*Paenibacillus polymyxa*), industrial (*Paenibacillus amylolyticus*) and medical applications (*P. peoriate*).

In the present study, we describe the taxonomic characterization of a novel strain, designated SK-3146^T, which appeared to be a member of the genus *Paenibacillus*. Strain SK-3146^T was cultured routinely on R2A agar plates at 30 °C and preserved as a suspension in R2A broth supplemented with 20 % (v/v) glycerol at −80 °C.

Genomic DNA of strain SK-3146^T was extracted with a commercial genomic DNA extraction kit (Solgent) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Im *et al.* [18]. A nearly full-length sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) or (http://www.ezbiocloud.net/eztaxon; [19]). Multiple alignments were performed using the CLUSTAL_X program [20]. Gaps were edited in the BioEdit program [21] and 1370 nucleotides were used for phylogenetic tree reconstruction. Evolutionary distances were

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Abbreviations: DAP, Diaminopimelic acid; DPG, Diphosphatidylglycerol; L, Lipid; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PL, Phospholipid; PME, Phospatidylmethylethanolamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SK- 3146^T is KX462877.

Three supplementary figures and one supplementary table are available with the online Supplementary Material.

calculated using the Kimura two-parameter model [22]. Phylogenetic trees were reconstructed with the neighbour-joining [23], maximum-parsimony [24] and maximum-likelihood methods by using the MEGA6 program [25] with bootstrap values based on 1000 replications [26].

The nearly complete 16S rRNA gene sequence of strain SK-3146^T (1465 nt) was determined and subjected to comparative analysis. Phylogenetic analysis using the maximum-likelihood method, based on 16S rRNA gene sequences, indicated that strain SK-3146^T clustered within the genus *Paenibacillus* (Fig. 1). Moreover, this relationship was also evident in phylogenetic trees based on the neighbour-joining method (Fig. S1, available in the online Supplementary Material) and maximum-parsimony method. The highest degree of sequence similarity determined was with *Paenibacillus yunnanensis* JCM 30953^T (98.6 %).

Based on 16S rRNA gene sequences and phylogenetic analysis, *P. yunnanensis* JCM 30953^T and *Paenibacillus vulneris* JCM 18268^T were selected as the closest neighbours of strain SK-3146^T and so were obtained from the Japan Collection of Microorganisms. They were then grown under the same conditions and used as reference strains in the majority of the subsequent phenotypic tests.

Sequence similarity calculations indicated that the most closely related strains to SK-3146^T were *P. yunnanensis* JCM 30953^T (98.6 %) and *P. vulneris* JCM 18268^T (98.0 %). Lower sequence similarities were obtained with *Paenibacillus chinjuensis* DSM 15045^T (96.9 %) and *Paenibacilllus rigui* KCTC 13282^T (96.8 %). The phylogenetic tree (Fig. 1) based on the maximum-likelihood algorithm showed that strain SK-3146^T clustered within the genus *Paenibacillus* and shared a branching node with *P. yunnanensis* JCM 30953^T. The tree topologies obtained using the other two algorithms (neighbour-joining and maximum-parsimony) were similar to that obtained when the maximum-likelihood algorithm was used (Fig. 1). Based on the EzTaxon-e server (16S rRNA gene sequence similarities), strain SK-3146^T did not show a high similarity to the type species *P. polymyxa* ATCC 842^T. Similarly, phylogenetic tree reconstruction indicated that the novel isolate had a distinct position with *P. yunnanensis* JCM 30953^T and *P. vulneris* JCM 18268^T within trees, which were then used as references for comparison with the novel isolate.

Cell shape, size, and the presence of flagella were observed under a Nikon light microscope (×1000 magnification) and transmission electron microscopy (LEO 912AB; Carl Zeiss)

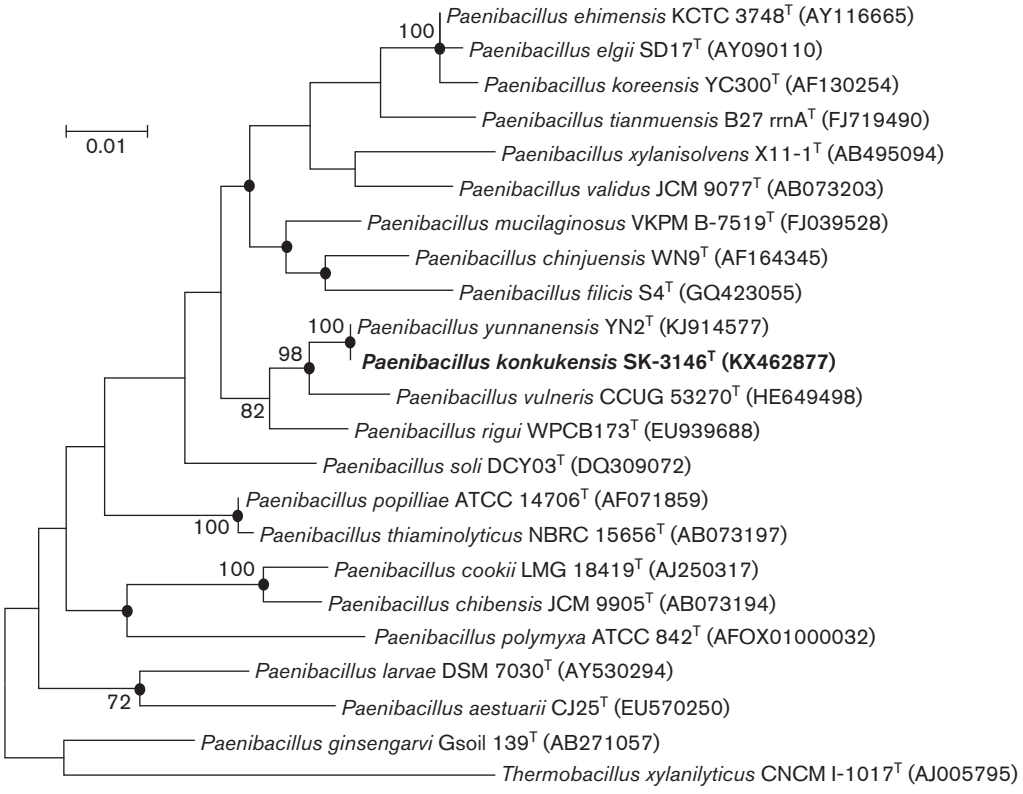

Fig. 1. Maximum-likelihood tree showing the phylogenetic relationships of strain SK-3146^T and members of the genus *Paenibacillus*. Bootstrap values greater than 70 % based on 1000 replications are shown at branching points. Filled circles indicate that the corresponding nodes were also obtained in trees reconstructed with the neighbour-joining and maximum-parsimony algorithms. *Thermobacillus xylanilyticus* CNCMI-1017^T was used as an outgroup. Scale bar, 0.01 substitutions per nucleotide position.

at 100 kV under standard operating conditions. Motility was tested on R2A broth supplemented with 0.2 % agar (w/v) [27]. Gram-staining was analyzed by using the non-staining method, as described by Buck [28]. Cell growth was monitored at different temperatures (0, 5, 10, 15, 18, 20, 25, 30, 35, 37, 40, 42, 45 and 50 °C). Similarly, the growth of strain SK-3146^T was tested on different media: R2A (Difco), trypticase soy agar (TSA; Difco), Luria-Bertani (LB; Difco), nutrient agar (NA; Difco), and MacConkey agar (Difco) at 30 °C for 7 days. Different initial pH values (4–10 at intervals of 0.5 pH units) were achieved by using the following buffers: citric acid/sodium citrate (pH 4.0–6.0), Na_2HPO_4/NaH_2PO_4 (pH 7.0–8.0), and Na_2CO_3/$NaHCO_3$ (pH 9.0–10.0), [29]. Salt tolerance was tested using trypticase soy broth supplemented with 0.5 to 6.0 % (w/v; with interval of 0.5 %) NaCl for 5 days at 30 °C. Oxidase activity was determined by using 1 % (w/v) *N*, *N*, *N*, *N*-tetramethyl-1,4-phenylenediamine reagent (bioMérieux), and catalase activity was determined from the production of bubbles from 3 % (v/v) H_2O_2 solution. Hydrolysis activity was tested by using the following substrates: starch (TSA containing 1 % starch [Difco]), casein (TSA containing 2 % skimmed milk [Difco]) [30], aesculin (TSA containing aesculin [0.3 % Sigma] and ferric citrate [0.2 % Fluka]), DNAse (DNAse agar medium [Sharlau]), gelatin [a medium containing 12 % gelatin, 0.3 % beef extract and 0.5 % peptone], and Tween 80 [TSA containing 1 % Tween 80 and 0.02 % $CaCl_2$]. Indole production was tested by using 1 % tryptone broth. Nitrate reduction was tested in nitrate broth with 0.2 % KNO_3 [31]. Xylan and cellulose degrading activity [32] were determined and evaluated after 5 days of incubation at 30 °C. Strain SK-3146^T was cultured on trypticase soy agar plates and incubated in a GasPak EZ (BD) for 14 days at 30 °C in order to check anaerobic growth. According to the manufacturer's instructions (API bioMerieux), enzyme production and carbon source utilization were determined using API ZYM, API ID 32 GN and API 20 NE. All the strips were incubated at 30 °C and recorded after 24 and 48 h.

Cells of strain SK-3146^T were Gram-stain-positive, rod shaped, aerobic and non-motile, (Fig. S2), and colonies were milky on R2A and NA agar media. Strain SK-3146^T produced an ellipsoidal terminal endospore in a swollen sporangium. Strain SK-3146^T was negative for indole production, starch, gelatin, xylan, Tween-80, DNase and cellulose hydrolysis, but positive for nitrate reduction and casein hydrolysis. The results of the physiological characterization, performed using methods described previously [33, 34], are given in Tables 1 and S1, as well as in the species description.

The DNA G+C content was determined by the method of Mesbah *et al.* [35] after the degradation of DNA to nucleosides by nuclease P1 and alkaline phosphatase enzymes. The subsequent separation of the nucleosides was measured by using a reversed-phase HPLC column (YMC-Triart C18; 4.6×250 mmx5 µm).

By using the fluorometric microplate method [36], DNA–DNA hybridization experiments were carried out in

Table 1. Differential phenotypic characteristics of strain SK-3146^T and the type strains of related species of the genus *Paenibacillus*

Strains: 1, SK-3146^T; 2, *P. yunnanensis* JCM 30593^T (present study); 3, *P. vulneris* JCM 18268^T (present study; [16]); 4, *P. rigui* KCTC 13282^T [42]; 5, *Paenibacillus soli* LMG 23604^T [43]; 6, *Paenibacillus filicis* S4^T[44]; 7, *P. chinjuensis* DSM 15045^T [45]; 8, *Paenibacillus validus* DSM 3037^T [46, 47]; 9, *Paenibacillus ehimensis* DSM 11029^T ([48]; Wu *et al.*, 2011); 10, *Paenibacillus koreensis* YC300^T [49]; 11, *P. polymyxa* [2, 50, 51]. +, Positive reaction; −, negative reaction; ND, not determined/ no data available; AF, Animal feed; PT, pu'er tea; NW, necrotic wound; W, water; S, soil; F, fern; C[1], chinju; C, compost; M, milky; S, shiny; C, creamy; LP, Light pink; GW, Greyish white; T, translucent.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Isolation source	AF	PT	NW	W	S	F	C[1]	ND	S	C	ND
Colony colour	M	ND	S	C	C	ND	LP	GW	C	T	ND
Motility	−	ND	−	+	−	+	+	+	+	+	
Temperature range for growth (°C)	18–45	ND	15–50	15–37	15–37	15–37	20–45	10–40	20–50	10–50	ND
pH range for growth	6–9.5	ND	5.5–11.5	5.0–8.0	5.0–8.0	5.5–9.0	5.5–8.5	5.6	5.8–6.3	ND	ND
Oxidase	+	ND	+	+	−	+	+	−	+	+	−
α-Glucosidase	+	+	+	+	−	+	+	W	+	+	
β-Glucosidase	+	+	+	+	−	−	ND	+	ND	+	
Growth at 50 °C	−	ND	+	+	−	−	−	+	+	+	−
Growth at 5 % (w/v) NaCl	−	ND	−	+	−	−	−	−	ND	+	−
Indole production	−	−	−	−	+	−	ND	−	−	−	−
Assimilation of:											−
L-Arabinose	+	+	+	+	+	−	−	−	+	+	ND
Inositol	+	+	+	−	−	−	ND	+	+	+	−
D-Mannitol	+	+	+	−	−	+	−	+	+	+	+
Salicin	+	+	+	−	+	ND	−	−	+	−	+
Isoprenoid quinone	MK-7, MK-6	MK-7	MK-7 MK-6	MK-7	MK-7	MK-7	MK-7	ND	MK-7	ND	MK-7
DNA G+C content (mol%)	53.8	51	ND	48.3	56.6	53.2	53	53–54	52.9–54.9	54	43–46

triplicate. The DNA–DNA hybridization values between strain SK-3146^T and *P. yunnanensis* JCM 30953^T and *P. vulneris* CCUG 53270T were 26.13±0.8 % and 38.7±0.6 %, respectively. This hybridization value is below the 70 % threshold proposed for species delineation [37], showing that strain SK-3146^T represents a distinct genomic species of the genus *Paenibacillus*. The DNA G+C content of strain SK-3146^T was 53.8 mol%, which is within the range for members of the genus *Paenibacillus*.

The polar lipids were extracted from 50 mg of freeze-dried cells, and examined by two-dimensional TLC and then identified as described by Minnikin *et al.* [38]. The isoprenoid quinones were extracted from 100 mg freeze-dried cells with chloroform/methanol (2 : 1 v/v), according to the method of Collins [39]. The crude quinone in *n*-hexane solution was purified using Sep-Pak Vac Silica Cartridges and subsequently analyzed by HPLC using a reversed-phase column (YMC-Triater C18; 4.6×250 mm×5 µm). Peptidoglycan analysis of strain SK-3146^T was analysed with the *meso*-DAP (standard), as described by Schleifer and Kandler [40], using TLC plates (Merck; TLC cellulose KGaA; 20×20 cm).

For fatty acid analysis, the cell mass of strain SK-3146^T and the closely related type strains, *P. yunnanensis* JCM 30953^T and *P. vulneris* JCM 18268^T, were grown on TSA agar at 28 °C for 48 h. The cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acid methyl esters were then analyzed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package [41].

The main polar lipids detected in strain SK-3146^T were diphosphatidylglycerol (DPG), and phosphatidylethanolamine (PE), while the minor polar lipids were phosphatidylglycerol (PG), phosphatidylmethylethanolamine (PME) one unknown lipid (L) and one unidentified phospholipid (PL) (Fig. S3). Polar lipid profiles of *P. vulneris* JCM 18268^T [16] and *P. rigui* [42] exhibited the same major compounds. The quinone system, consisting of menaquinone MK-7 (80 %) and MK-6 (20 %), supported the affiliation of strain SK-3146^T to the genus *Paenibacillus*. The peptidoglycan of strain SK-3146^T contained *meso*-diaminopimelic. The fatty acid profile comprised mainly iso- and anteiso-branched fatty acids and was very similar to those of the most closely related species of the genus *Paenibacillus*. The detailed fatty acid profile obtained from cells grown on TSA medium after 48 h incubation at 30 °C is shown in Table 2. The major fatty acids of strain SK-3146^T were anteiso-$C_{15:0}$ (65.0 %) and $C_{16:0}$ (15.0 %); lesser amounts of anteiso-$C_{17:0}$ (6.5 %),

Table 2. Cellular fatty acid profiles (as percentages of total fatty acids) of strain SK-3146^T and related species of the genus *Paenibacillus*

Strains: 1, Sk-3641^T; 2, *P. yunnanensis* JCM 30593^T; 3, *P. vulneris* JCM 18268^T (data in parentheses from Glaeser *et al.* [16]); 4, *P. rigui* KCTC 13282^T [42]; 5, *P. soli* LMG 23604^T [43]; 6, *P. filicis* S4^T [44]; 7, *P. chinjuensis* DSM 15045^T [45]; 8, *P. validus* DSM 3037^T [46, 47]; 9, *P. ehimensis* DSM 11029^T [48]; 10, *P. koreensis* YC300^T [49]; 11, *P. polymyxa* DSM 36^T [52]. All strains were cultured on TSA agar at 30 °C for 48 h. –, Not determined/no data available. Data for strains 1, 2 and 3 are from the present study.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
Saturated											
$C_{12:0}$	–	1.4	1.1	–	–	–	–	–	–	–	–
$C_{14:0}$	–	0.8	1.1	–	1.7	1.9	–	1.7	1.0	2.5	0.7
$C_{15:0}$	–	–	–	10.8	6.9	–	4.5	1.5	1.2	–	0.5
$C_{16:0}$	5.8	8.2	8.3	3.6	10.5	9.4	5.3	7.2	7.1	14.4	9.1
$C_{18:0}$	–	–	–	–	4	–	–	–	–	–	1.1
Branched											
iso-$C_{14:0}$	3.8	4.1	3.8	2.9	2.3	4.4	2.2	2.3	1.8	–	0.6
iso-$C_{15:0}$	3.3	4.1	3.6	9.3	3.6	4.8	5	15.1	8.1	5.3	5.5
anteiso-$C_{15:0}$	**65.0**	**60.1**	**61**	**65.2**	**57.6**	**57.5**	**66.1**	**52.7**	**52.9**	**51.8**	**49.9**
iso-$C_{16:0}$	**15.7**	**14.2**	**12.1**	**5.4**	**5.6**	**11.3**	**7**	**6.3**	**8.6**	**5.6**	**7.7**
iso-$C_{17:0}$	–	0.7	1.3	2.0	–	2.3	1.6	3.3	3.3	3.2	7.0
anteiso-$C_{17:0}$	6.5	6.4	5.7	3.1	2.7	5.2	6.1	4.6	8	7.9	16.7
Unsaturated											
$C_{16:1}\omega 7c$ alcohol	–	–	–	–	–	–	–	–	1.2	–	–
$C_{16:1}\omega 11c$	–	–	–	–	–	–	–	2.6	5.2	9.3	–
$C_{18:1}\omega 9c$	–	–	–	–	2.3	–	–	–	–	–	–
Summed features*											
4; $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$	–	–	–	–	1.4	–	–	–	–	–	–
7; $C_{18:1}\omega 7c/\omega 9t/\omega 12t$	–	–	–	–	1.5	–	–	–	–	–	–

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Only fatty acids showing relative amounts >1 % of the total are shown.

$C_{16:0}$ (5.8 %), iso-$C_{15:0}$ (3.3 %), and iso-$C_{14:0}$ (3.8 %) were also found.

Therefore, on the basis of the data presented, we consider that strain SK-3146^T represents a novel species of the genus *Paenibacillus* for which the name *Paenibacillus konkukensis* sp. nov. is proposed.

DESCRIPTION OF *PAENIBACILLUS KONKUKENSIS* SP. NOV.

Paenibacillus konkukensis (kon.kuk.en′sis. N.L. mac. adj. *konkukensis* named after Konkuk University where this taxonomic study was performed).

Cells are Gram-stain-positive, aerobic and rod-shaped (width, 0.5–1.0 µm; length, 1.5–2.5 µm). The ellipsoidal terminal endospores are formed in swollen sporangia. Colonies after 48 h of incubation on R2A are circular, convex and milky, with an average diameter of 1.2–2.5 mm. No growth on Luria–Bertani agar (Difco) and DNase agar (Difco). Optimum temperature for growth is 30 °C; growth occurs at 18–45 °C, but not at 15 °C or 50 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 6.0–9.5. Grows in the presence of 1–3 % NaCl (w/v, but not at 4 %), when tested in R2A broth. In API ZYM, 32GN and 20NE test strips, positive for aesculin hydrolysis, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, nitrate reduction, arginine and urea. Negative for indole production, α-chymotrypsin and α-mannosidase. The following compounds are used as a sole source of carbon and energy: D-ribose, inositol, sucrose, maltose, L-arabinose, D-mannitol, salicin, melibiose and gluconate, while D-glucose, D-mannose, capric acid, adipic acid, phenyl acetate, L-fucose, D-sorbitol, propionic acid, valeric acid, 2-ketogluconate, 4-hydroxy-benzoate, L-proline, L-rhamnose, itaconic acid, suberate, sodium malonate, sodium acetate, lactic acid, L-alanine, 5-ketogluconate, glycogen and L-serine are not utilized. The quinone system is composed of menaquinone MK-7 and MK-6 (80 : 20). Major polar lipids are diphosphatidylglycerol and phosphatidylethanolamine. Predominant fatty acids are anteiso-$C_{15:0}$ and iso-$C_{16:0}$, with lesser amounts of anteiso-$C_{17:0}$, iso-$C_{15:0}$, iso-$C_{14:0}$ and $C_{16:0}$ detected. The diagnostic cell wall peptidoglycan amino acid is *meso*-diaminopimelic acid.

The type strain, SK-3146^T (=KACC 18876^T=LMG 29568^T), was isolated from animal feed, in the Republic of Korea. The DNA G+C content of the type strain is 53.8 mol%.

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Conflicts of interest
The authors declare that there are no conflict of interest.

Ethical statement
This research is the author's original work and has not been submitted elsewhere for publication. All authors listed performed the data analysis and manuscript writing and "This article does not contain any studies with human participants or animals performed by any of the authors. Moreover, all authors read and approved the final manuscript.

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