Knoellia flava sp. nov., isolated from pig manure

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A Gram-positive, aerobic, non-spore-forming actinobacterial strain, designated strain TL1^T, was isolated from pig manure in Wuhan, China. The cell wall peptidoglycan contained meso-diaminopimelic acid. The major polar lipids were phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol. The predominant menaquinone was MK-8(H₄). The major fatty acids (>10%) were iso-C_{16:0}, iso-C_{15:0} and C_{17:1}ω8c. The genomic DNA G + C content was 70.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain TL1^T was most closely related to the type strains of *Knoellia sinensis* (98.5%), *Knoellia subterranea* (98.2%) and *Knoellia aerolata* (96.9%). DNA–DNA relatedness values of strain TL1^T with the type strains of *K. sinensis* and *K. subterranea* were 27.3 and 34.0%, respectively. Comparison of phenotypic, chemotaxonomic and phylogenetic characteristics among strain TL1^T and related organisms revealed that the isolate represents a novel species of the genus *Knoellia*, for which the name *Knoellia flava* sp. nov. is proposed; the type strain is TL1^T (=CGMCC 1.10749^T=KCTC 19810^T).

The family *Intrasporangiaceae* was first described by Stackebrandt *et al.* (1997), and later emended by Stackebrandt & Schumann (2000) and Zhi *et al.* (2009). Currently, it contains 19 genera with validly published names, which have been divided into three groups based on the diagnostic diamino acids in the cell-wall peptidoglycan [eight genera with meso-diaminopimelic acid (DAP), seven with LL-DAP and four with L-ornithine] (Lee, 2006; Wang *et al.*, 2009). The genus *Knoellia* belongs to the first group and was proposed by Groth *et al.* (2002) based on the studies of two species, *Knoellia sinensis* and *Knoellia subterranea*, isolated from soil in Reed Flute Cave, Guilin, China. Weon *et al.* (2007) reported another species, *Knoellia aerolata*, which was isolated from air. At the time of writing, the genus *Knoellia* contained only three species, with the following typical characteristics: Gram-positive, aerobic, non-spore-forming, irregular rods or cocci, containing MK-8(H₄) as the major menaquinone and meso-DAP in the cell-wall peptidoglycan (Groth *et al.*, 2002; Weon *et al.*, 2007).

During the course of an investigation of cultivable bacteria from pig manure, bacteria were isolated from a pig manure sample collected from a hogger in Huazhong Agricultural University (114° 35' E 30° 48' N), Wuhan, central PR China. For isolation, 10 g sample was mixed thoroughly

with 90 ml 0.85% NaCl solution, plated onto 10% tryptic soy broth (0.1 × TSB; Difco) agar plates and incubated at 28 °C for 1 week. A total of seven bacterial strains were isolated and pre-identified as *Bacillus* (three strains), *Klebsiella* (two strains), *Knoellia* (one strain, TL1^T) and *Paenibacillus* (one strain) based on partial 16S rRNA gene sequence analysis. Here, strain TL1^T was studied using a polyphasic approach in order to determine its taxonomic position.

Strain TL1^T was routinely cultured on R agar (g per 100 ml: bacto peptone, 1; yeast extract, 0.5; Casamino acids, 0.5; beef extract, 0.2; malt extract, 0.5; glycerol, 0.2; MgSO₄·7H₂O, 0.1; Tween 80, 0.005) at 28 °C for 3 days. Phenotypic characterizations were performed following the recommended minimal standards for describing new genera and species of the suborder *Micrococcineae* (Schumann *et al.*, 2009). Growth was tested at 0, 4, 15, 20, 28, 37 and 42 °C. Colony morphologies were observed on R and R2A (g per 100 ml: yeast extract, 0.05; proteose peptone, 0.05; Casamino acids, 0.05; glucose, 0.05; soluble starch, 0.05; sodium pyruvate, 0.03; dipotassium phosphate, 0.03; magnesium sulphate, 0.005) plates. Cell morphologies were observed by light and scanning electron microscopy after various incubation times. NaCl tolerance was determined with 0–6% (w/v) NaCl in R medium at 28 °C. The pH range (4–10) for growth was determined at 28 °C. Anaerobic growth was determined by incubation in an anaerobic chamber (Mitsubishi Gas Chemical Co.) at 28 °C. Gram staining was determined using the method described by Dussault (1955) and tested by the KOH lysis method (Ryu, 1938). Hydrolysis of casein, aesculin, gelatin,

Abbreviations: DAP, diaminopimelic acid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TL1^T is HQ401007.

Four supplementary figures are available with the online version of this paper.

L-tyrosine, urea, starch, Tween 80, Tween 20, chitin from crab shells and carboxymethyl-cellulose was performed as described by Cowan & Steel (1965). Nitrate reduction was tested as described by Lányi (1987). Methyl red and Voges–Proskauer tests, and determination of H₂S and indole production, and catalase and oxidase activities were carried out as described by Smibert & Krieg (1994). Antibiotic susceptibility tests were performed by spreading bacterial suspensions on culture plates and applying filter-paper discs containing different antibiotics. Other physiological/biochemical properties and enzyme activities were examined using API 20NE, ID 32GN and API ZYM systems (bioMérieux) according to the manufacturer's instructions.

A nearly completed 16S rRNA gene sequence was amplified as described by Zou & Wang (2010) and compared with sequences available in NCBI/GenBank using BLASTN searches. The 16S rRNA gene sequence of strain TL1^T was aligned with the corresponding sequences of members of the family *Intrasporangiaceae* using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic analysis was performed using MEGA4.0 (Tamura *et al.*, 2007) and the PHYML online web server (Guindon *et al.*, 2005). Distances and clustering were determined using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods with bootstrap analyses based on 1000 replications. DNA–DNA hybridization

was performed by the thermal denaturation and renaturation method (Huß *et al.*, 1983).

Polar lipids were extracted and analysed as described by Tindall (1990) and Ventosa *et al.* (1993). Peptidoglycan was analysed by the method of Schleifer & Kandler (1972). The DNA G+C content of strain TL1^T was determined by HPLC according to the method of Mesbah *et al.* (1989). For whole-cell fatty acid analysis, strain TL1^T and three reference strains were grown on R2A agar at 28 °C for 3 days and analysed by GC (Hewlett Packard 6890) according to the standard protocol of the Sherlock Microbial Identification System (MIDI Sherlock version 4.5; MIDI database TSBA40 4.10) (Kroppenstedt, 1985; Sasser, 1990). Respiratory quinones were extracted and identified by HPLC as described by Xie & Yokota (2003).

Cells of strain TL1^T were Gram-positive, non-motile, non-spore-forming, irregular rods and cocci. Colonies were circular, smooth and convex on R medium. Colonies of strain TL1^T were light yellow on R agar and yellow on R2A agar, whereas colonies of the other *Knoellia* species were all creamy white on both R agar and R2A agar plates (see Supplementary Fig. S1, available in IJSEM Online). As observed in the three reference strains, strain TL1^T exhibited a rod–coccus growth cycle; cells were mostly irregular rod shapes after incubation for 1 day and mostly

Table 1. Differential phenotypic characteristics of strain TL1^T and the type strains of members of the genus *Knoellia*

Strains: 1, TL1^T; 2, *K. sinensis* KCTC 19936^T; 3, *K. subterranea* KCTC 19937^T; 4, *K. aerolata* DSM 18566^T. All data are from this study. +, Positive; –, negative; w, weak reaction.

Characteristic	1	2	3	4
Colour on:				
R agar	Light yellow	Creamy white	Creamy white	Creamy white
R2A agar	Yellow	Creamy white	Creamy white	Creamy white
NaCl range (% w/v)	0–5.0	0–4.0	0–4.0	0–2.0
Growth at 37 °C	+	–	+	+
Hydrolysis of:				
Casein	+	+	+	–
Tyrosine	–	+	+	–
API 20NE/API 32GN results:				
Arginine dihydrolase	w	–	–	–
D-Mannose	+	–	+	+
D-Mannitol	+	–	+	+
N-Acetylglucosamine	–	–	+	+
Potassium gluconate	–	–	–	+
Adipic acid	–	w	–	–
Malic acid	+	–	+	–
D-Ribose	–	–	+	–
Inositol	–	–	–	+
Lactic acid	+	w	+	–
L-Alanine	+	–	+	+
L-Serine	+	+	+	–
D-Sorbitol	+	–	+	+
L-Histidine	+	+	+	–

cocci after incubation for 3 days (Supplementary Fig. S2, available in IJSEM Online). Detailed results of the morphological, physiological and biochemical characteristics are given in the species description. Strain TL1^T showed some characteristics that were typical of the other *Knoellia* species but many differences were also observed. The main phenotypic differences between strain TL1^T and type strains of the genus *Knoellia* are shown in Table 1.

The 1484 bp 16S rRNA gene sequence of strain TL1^T was similar to those of members of the *Intrasporangiaceae*, particularly to those of the genus *Knoellia*. Strain TL1^T had highest 16S rRNA gene sequence similarities to *K. sinensis* DSM 12331^T (98.5%), *K. subterranea* DSM 12332^T (98.2%), *K. aerolata* 5317S-21^T (96.9%), *Janibacter anophelis* CCUG 49715^T (97.1%) and *Janibacter melonis* DSM 16063^T (97.1%). A phylogenetic tree constructed using the neighbour-joining algorithm revealed that strain TL1^T clustered with the three species of the genus *Knoellia* and was separated from members of the genus *Janibacter* and other genera of the family *Intrasporangiaceae* (Fig. 1). Analyses using the maximum-parsimony and the maximum-likelihood algorithms showed similar results (Supplementary Fig. S3a, b, available in IJSEM Online). DNA–DNA relatedness values of strain TL1^T with the type strains of *K. sinensis* and *K. subterranea* were 27.3 and 34.0%, respectively. These values were significantly lower than the threshold value (70%) suggested for the delineation of novel species (Stackebrandt & Goebel, 1994).

The major menaquinone of strain TL1^T was MK-8(H₄). The cell wall peptidoglycan contained *meso*-DAP as the

diagnostic di-amino acid. These two characters were identical to those reported for the three species within the genus *Knoellia* (Groth *et al.*, 2002; Weon *et al.*, 2007). The polar lipid profile of strain TL1^T included PE, PI, DPG and a small amount of unknown phospholipid(s) (PL) (Supplementary Fig. S4, available in IJSEM Online). The other three species of the genus *Knoellia* also contained PE, PI and DPG as the major polar lipids; in addition, they all possessed very small amounts of phosphatidylglycerol (PG) (Groth *et al.*, 2002; Weon *et al.*, 2007), which was not detected in strain TL1^T. The DNA G + C content of strain TL1^T was 70.9 mol%. Major cellular fatty acids (>10%) were iso-C_{16:0} (33.6%), iso-C_{15:0} (15.9%) and C_{17:1ω8c} (11.5%) on R2A medium (details are shown in Table 2), which were very similar to those observed in strains of the genus *Knoellia* (Weon *et al.*, 2007). Although strain TL1^T exhibited 97.1% 16S rRNA gene sequence similarities to strains of the two *Janibacter* species studied, phylogenetic analyses showed that they belonged to two separate clusters. In addition, the major polar lipids and fatty acid contents clearly distinguished strain TL1^T from members of the genus *Janibacter*. Members of the genus *Knoellia* contain PE as a major polar lipid and iso-C_{15:0} as a major fatty acid, whereas species of the genus *Janibacter* lacked PE and had a much lower amount of iso-C_{15:0} (Martin *et al.*, 1997; Groth *et al.*, 2002; Lang *et al.*, 2003; Yoon *et al.*, 2004; Kämpfer *et al.*, 2006; Kageyama *et al.*, 2007; Weon *et al.*, 2007; Shivaji *et al.*, 2009) (Table 2). There were differences in the proportions of some fatty acids when the strains were grown on R2A and TSB media. Groth *et al.* (2002)

Fig. 1. A neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain TL1^T among closely related strains. Bootstrap values ($\geq 50\%$) based on 1000 replications are shown at branch nodes. Bar, 0.005 substitutions per nucleotide.

Table 2. Chemotaxonomic characteristics of strain TL1^T and the type strains of members of the genera *Knoellia* and *Janibacter*

Taxa: 1, TL1^T; 2, *K. sinensis* KCTC 19936^T; 3, *K. subterranea* KCTC 19937^T; 4, *K. aerolata* DSM 18566^T; 5, *J. melonis* CM2104^T; 6, *J. anophelis* CCUG 49715^T; 7, *J. limosus* DSM 11140^T; 8, *J. brevis* DSM 13953^T; 9, *J. terrae* CS12^T. Except where marked, data for 1–4 are from this study (cells grown on R2A plates for 3 days); data for 5, 6 and 7–9 are from Yoon *et al.* (2004), Kämpfer *et al.* (2006) and Lang *et al.* (2003), respectively. —, <1 % or not detected; NA, not available.

Characteristic	1	2	3	4	5	6	7	8	9
Fatty acid:									
iso-C _{14:0}	8.3	6.5	10.9	1.7	2.8	4.7	—	—	1.1
C _{15:0}	3.1	—	1.7	—	12.1	3.3	—	—	2.4
iso-C _{15:0}	15.9	8.7	11.7	19.9	5.1	4.4	—	3.72	—
anteiso-C _{15:0}	—	—	3.2	5.6	1.2	—	—	—	—
C _{16:0}	1.2	1.4	1.8	3.6	2.5	2.5	2.1	1.6	—
iso-C _{16:0}	33.6	47.6	34.5	13.9	16.7	32.9	12.2	30.5	38.1
iso-C _{16:1} H	—	5.3	1.4	—	—	—	—	—	2.1
C _{17:0}	5.5	—	2.8	2.3	13.4	16.4	17.8	2.2	3.5
C _{17:0} 10-methyl	3.7	7.1	3.9	1.2	—	2.1	2.8	2.7	6.0
C _{17:1} ω8c	11.5	1.1	6.9	4.7	20.0	—	26.2	20.9	11.9
iso-C _{17:0}	2.1	4.5	4.4	4.9	4.1	3.3	1.5	6.6	3.9
iso-C _{17:1} ω9c	1.7	11.4	4.8	6.2	—	—	—	2.1	—
anteiso-C _{17:0}	—	—	2.4	15.7	2.2	2.0	—	2.0	5.7
anteiso-C _{17:1} ω9c	—	—	—	1.9	—	—	—	—	—
C _{18:0}	—	—	—	2.1	1.5	2.7	3.9	—	—
C _{18:1} ω9c	2.1	—	—	9.0	7.6	3.9	17.5	15.8	13.3
iso-C _{18:0}	1.2	—	2.3	—	1.1	3.2	3.4	1.3	—
C _{19:0}	—	—	—	—	—	—	1.9	—	—
Summed feature 3*	1.6	—	3.4	1.9	2.1	—	—	—	—
Summed feature 6*	2.5	1.8	—	—	3.7	—	—	3.7	2.32
Polar lipids†	DPG, PI, PE, PL	DPG, PI, PE, PL, PG ^a	DPG, PI, PE, PL, PG ^a	DPG, PI, PE, PL, PG ^b	NA	NA	DPG, PI, PG ^c	DPG, PI, PG ^d	DPG, PI, PG ^e
DNA G + C content (mol%)†	70.9	68 ^a	69 ^a	73.0 ^b	73	NA	70 ^c	72 ^d	69 ^e

*Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; summed feature 6 comprises C_{19:1}ω9c and/or C_{19:1}ω11c.

†Data from: a, Groth *et al.* (2002); b, Weon *et al.* (2007); c, Martin *et al.* (1997); d, Imamura *et al.* (2000); and e, Yoon *et al.* (2000).

showed that *K. sinensis* and *K. subterranea* contained iso-C_{15:0}, iso-C_{17:0}, iso-C_{16:0} and anteiso-C_{17:0} as major fatty acids (>10 %) on TSB media. However, Weon *et al.* (2007) showed that *K. aerolata* had iso-C_{16:0}, C_{17:1}ω8c and iso-C_{15:0} as the major fatty acids (>10 %) on R2A media. In this study, we reanalysed the fatty acids for strains of the three *Knoellia* species together with strain TL1^T on R2A media and the results were very similar to those reported by Weon *et al.* (2007).

Therefore, on the basis of the distinctive phenotypic, chemotaxonomic and phylogenetic traits, strain TL1^T represents a novel species in the genus *Knoellia*, for which the name *Knoellia flava* sp. nov. is proposed.

Description of *Knoellia flava* sp. nov.

Knoellia flava (fla'va. L. fem. adj. *flava* yellow, referring to colony colour after incubation on R2A at 28 °C for 3 days).

Cells are Gram-positive, non-motile, non-spore-forming, non-acid-fast, irregular rods (0.6 × 1.0–1.9 µm) and cocci (0.5–1.1 µm diameter). Colonies are yellow, circular, smooth and convex on R2A agar, and light yellow, circular, smooth and convex on R agar. Grows aerobically at 4–37 °C (optimum growth at 28 °C), but not at 0 °C or 42 °C. Anaerobic growth is not observed. Grows at pH 5–9, with optimum growth at pH 7.0. In R medium, tolerates up to 5 % NaCl (optimum growth at 0 % NaCl). Grows on R agar, R2A agar, LB agar and TSB agar, but not on MacConkey agar. Catalase-positive and oxidase-negative. Tween 80, Tween 20, starch, gelatin, aesculin and casein are hydrolysed, but chitin, carboxymethyl-cellulose, L-tyrosine and urea are not hydrolysed. Positive for the production of H₂S, nitrate reduction and β-galactosidase, but negative for indole production, glucose fermentation, and Voges–Proskauer and methyl red tests. A weak reaction is detected for arginine dihydrolase. According to results from API 20NE and API ID 32GN test strips, D-glucose, D-mannose, D-mannitol, maltose, malic acid, sucrose, D-sorbitol, propionic acid, valeric acid, glycogen, salicin, sodium acetate, lactic acid, L-alanine, L-serine, L-histidine, 3-hydroxybutyric acid and L-proline are assimilated, but L-arabinose, N-acetylglucosamine, potassium gluconate, capric acid, adipic acid, trisodium citrate, phenylacetic acid, L-rhamnose, D-ribose, inositol, itaconic acid, suberic acid, sodium malonate, potassium 5-ketogluconate, 3-hydroxybenzoic acid, melibiose, L-fucose, potassium 2-ketogluconate and 4-hydroxybenzoic acid are not assimilated. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase activities are detected by the API ZYM system, but not α-chymotrypsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Weak reactions are detected for lipase (C14) and trypsin. Resistant to (µg) penicillin (10), trimethoprim (5), ampicillin (10) and nalidixic acid (30). Sensitive to (µg) amoxicillin (20), cefotaxime (30), chloramphenicol (30),

kanamycin (30), polymyxin B (25), cefoxitin (30), streptomycin (10), erythromycin (15), nitrofurantoin (30), neomycin (30), novobiocin (30) and tobramycin (10). Major cellular fatty acids (>10 %) are iso-C_{16:0}, iso-C_{15:0} and C_{17:1}ω8c on R2A medium. The major polar lipids are PE, PI and DPG. The cell wall peptidoglycan type is *meso*-DAP. The predominant menaquinone is MK-8(H₄).

The type strain is TL1^T (=CGMCC 1.10749^T=KCTC 19810^T), isolated from pig manure in Wuhan, central PR China. The DNA G+C content of the type strain is 70.9 mol%.

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