Ornithinimicrobium kibberense sp. nov., isolated from the Indian Himalayas

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A buff-yellow-pigmented bacterium, strain K22-20^T, which was isolated from a cold desert of the Indian Himalayas, was subjected to a polyphasic taxonomic study. Phenotypic and chemical properties of strain K22-20^T were consistent with its classification in the genus *Ornithinimicrobium*. The major fatty acids of the strain were iso-C_{17:1ω9c} (*cis*-15-methyl 7-hexadecenoic acid), iso-C_{15:0} (13-methyl tetradecanoic acid), iso-C_{16:0} (14-methyl pentadecanoic acid) and iso-C_{17:0} (15-methyl hexadecanoic acid). The G + C content of the genomic DNA was 71 mol%. According to 16S rRNA gene sequence analysis, strain K22-20^T was closely related to *Ornithinimicrobium humiphilum* HKI 0124^T (97.7 %). However, genomic relatedness between strain K22-20^T and *O. humiphilum* MTCC 6406^T, as revealed by DNA–DNA hybridization, was 64.5 %. Based on the polyphasic data, strain K22-20^T (=MTCC 6545^T=DSM 17687^T=JCM 12763^T) represents a novel species of the genus *Ornithinimicrobium*, for which the name *Ornithinimicrobium kibberense* sp. nov. is proposed.

The suborder *Micrococcineae* Stackebrandt *et al.* 1997 currently consists of the following genera, which contain ornithine as diagnostic amino acid in their peptidoglycan: *Cellulomonas* Bergey *et al.* 1923, *Curtobacterium* Yamada and Komagata 1972, *Rarobacter* Yamamoto *et al.* 1988, *Microbacterium* Orla-Jensen 1919 emend. Takeuchi and Hatano 1998, *Ornithinococcus* Groth *et al.* 1999, *Salana* von Wintzingerode *et al.* 2001, *Ornithinimicrobium* Groth *et al.* 2001, *Rhodoglobus* Sheridan *et al.* 2003, *Arsenicicoccus* Collins *et al.* 2004 and *Serinicoccus* Yi *et al.* 2004. At the time of writing, the genus *Ornithinimicrobium* consists of only one species, *Ornithinimicrobium humiphilum*. In this study, a novel strain is described, K22-20^T, which was isolated from a soil sample collected from Lahaul-Spiti valley in the Indian Himalayas.

Strain K22-20^T was isolated by the dilution-plate technique on tryptone soya agar medium (TSA; HiMedia) and maintained as glycerol stocks at –70 °C. The reference strain *Ornithinimicrobium humiphilum* MTCC 6406^T was taken from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Strain K22-20^T and *O. humiphilum* MTCC 6406^T were grown on TSA at 28 °C and morphological and physiological properties were analysed. Colony and cell morphology were studied according to standard methods (Murray *et al.*, 1994). Gram reaction was

determined using the HiMedia Gram Staining kit according to the manufacturer's instructions. Physiological tests such as growth at different temperatures, pH and NaCl concentrations were examined by growing the strain on basal TSA. Catalase and urea hydrolysis were determined as described by Cowan & Steel (1965). Hydrolysis of casein, gelatin and starch, hydrogen sulphide production, methyl red and Voges–Proskauer tests and the presence of oxidase were determined as described by Smibert & Krieg (1994). Nitrate reduction and indole production were determined as described by Claus & Berkeley (1986). Utilization of various carbon sources was tested according to Reddy *et al.* (2003) and also using Biolog GP2 MicroPlates. The plates were used according to the manufacturer's instructions except that TSA medium was used instead of Biolog Universal Growth agar medium. Inoculated plates were incubated for 24 h and results were read with a MicroPlate Reader using Microlog 4.2 software. Acid production from various sugars was tested as described by Smith *et al.* (1952). Antibiotic susceptibility was tested by placing antibiotic discs (HiMedia) on Mueller–Hinton agar plates seeded with suspensions of strain K22-20^T.

Freeze-dried cells for chemotaxonomic analysis were prepared following growth of the strain in shake flasks containing tryptone soya broth (TSB; HiMedia) for 4 days at 28 °C. Whole-cell sugars were determined as described by Stanek & Roberts (1974). Peptidoglycan structure was determined using hydrolysates of purified cell walls according to Schleifer (1985). Amino acids and peptides were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Ornithinimicrobium kibberense* strain K22-20^T is AY636111.

separated by two-dimensional ascending TLC as described by Schleifer & Kandler (1972) with the modification that TLC on cellulose sheets (Merck 5577) was used instead of paper chromatography. Phospholipids and menaquinones were extracted and analysed as described by Minnikin *et al.* (1984). The glycolic acid content of the bacterial cell wall was determined by the colour reaction method of Uchida *et al.* (1999). For cellular fatty acid analysis, cells were grown on TSA at 28 °C for 36 h and fatty acid methyl ester analysis was performed with the Sherlock Microbial Identification System (MIDI) as described previously (Pandey *et al.*, 2002). DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987). The genomic DNA G + C content was determined spectrophotometrically (Lambda35; Perkin Elmer) using the thermal denaturation method (Mandel & Marmur, 1968).

The chromosomal DNA of strain K22-20^T was isolated according to Rainey *et al.* (1996). The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTTGATCCTGG-CTCAG-3') and 1500r (5'-AGAAAGGAGGTGATCCAGC-CA-3'). The amplified DNA fragment was separated on 1 % agarose gel, eluted from the gel and purified using a QIAquick gel extraction kit (Qiagen). The purified PCR product was sequenced with four forward and three reverse primers, namely 8-27f, 357f (5'-CTCCTACGGGAGGC-AGCAG-3'), 704f (5'-TAGCGGTGAAATGCGTAGA-3'), 1114f (5'-GCAACGAGCGCAACC-3'), 685r (5'-TCTAC-GCATTTCACCGCTAC-3'), 1110r (5'-GGGTTGCGCTC-GTTG-3') and 1500r (*Escherichia coli* numbering system). The 16S rRNA gene sequence was determined by the dideoxy chain-termination method with the Big-Dye terminator kit using an ABI 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequence of strain K22-20^T generated in this work (1488 bases) was aligned with 16S rRNA gene sequences of *O. humiphilum* and other members of the family *Intrasporangiaceae* and suborder *Micrococccineae*. A sequence similarity search was done using GenBank BLASTN (Altschul *et al.*, 1997). Sequences of closely related taxa were retrieved and aligned using the program CLUSTAL_X (Thompson *et al.*, 1997) and the alignment was corrected manually. For neighbour-joining analysis (Saitou & Nei, 1987), distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985).

Detailed test reactions are presented in the species description. Based on biochemical characteristics, strain K22-20^T differed from the closely related species *O. humiphilum* (Table 1). Most of the chemotaxonomic properties (presented in the species description) were typical of members of the genus *Ornithinimicrobium*. Cellular fatty acids differed quantitatively between strain K22-20^T and the type strain of *O. humiphilum* (Table 2). To determine the phylogenetic relationships of strain K22-20^T, the 16S rRNA gene sequence (1488 bp) was compared with those of type strains of species of the genera retrieved from GenBank. Results revealed that

Table 1. Differential phenotypic characteristics of strain K22-20^T and *O. humiphilum* MTCC 6406^T

All data are from the present study. Both strains are Gram-positive, do not produce endospores, are non-acid-fast and do not produce acid from L-arabinose, cellobiose, salicin, D-glucose, D-melibiose, D-lactose, inulin, D-mannose, L-rhamnose or D-xylose. Both strains are positive in the Biolog GP2 MicroPlate tests for dextrin, α -D-glucose, maltose, maltotriose, D-mannose, methyl α -D-glucoside, D-ribose, sucrose, D-trehalose, D-xylose, acetic acid, pyruvic acid, succinic acid, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, AMP and TMP; both are negative for α - and β -cyclodextrin, inulin, Tweens 40 and 80, N-acetyl-D-glucosamine, N-acetyl- β -D-mannosamine, amygdalin, L-arabinose, L-fucose, D-galacturonic acid, *myo*-inositol, α -D-lactose, lactulose, D-mannitol, D-melezitose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, 3-methylglucose, methyl β -D-glucoside, methyl β -D-mannoside, L-rhamnose, sedoheptulosan, D-sorbitol, D-tagatose, xylitol, α - and γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D- and L-malic acid, succinamic acid, N-acetyl-L-glutamic acid, D-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglyutamic acid, L-serine, putrescine, 2,3-butanediol, D-fructose 6-phosphate, α -D-glucose 1-phosphate, D-glucose 6-phosphate and DL- α -glycerol phosphate. Both grow in 2 and 4 % NaCl, but not in 8 % NaCl. Neither strain grows at 4 or 55 °C. Both strains are negative for methyl red and Voges–Proskauer tests, indole, hydrogen sulphide production and oxidase. Both are sensitive to colistin (10 µg per disc) and polymyxin B (300 µg per disc).

Characteristic	K22-20 ^T	MTCC 6406 ^T
Colony colour*	BY	LY
Acid production from:		
D-Fructose	+	–
D-Raffinose	+	–
Trehalose	+	–
Maltose	+	–
Sucrose	+	–
Growth at 42 °C	–	+
Growth at pH 9.0	+	–
Growth on 7 % NaCl	+	–
Nitrate reduction	+	–
Utilization of:		
Acetate	+	–
Citrate	+	–
Benzoate	+	–
Utilization as sole carbon source†		
Glycogen	+	–
Mannan	+	–
D-Arabitol	+	–
Arbutin	+	–
D-Cellobiose	+	–
D-Fructose	+	–
D-Galactose	+	–
Gentiobiose	+	–
D-Gluconic acid	+	–
Palatinose	+	–

Table 1. cont.

Characteristic	K22-20 ^T	MTCC 6406 ^T
D-Psicose	+	—
D-Raffinose	+	—
Salicin	+	—
Stachyose	+	—
Turanose	+	—
β-Hydroxybutyric acid	+	—
α-Ketovaleic acid	—	+
Succinic acid monomethyl ester	—	+
Propionic acid	—	+
L-Alaninamide	+	—
Susceptibility to:		
Lincomycin (2 µg per disc)	+	—
Triple sulphas (300 µg per disc)	+	—

*BF, Buff yellow; LY, lemon yellow.

†Using Biolog GP2 MicroPlates.

strain K22-20^T is a member of the suborder *Micrococcineae* (Stackebrandt *et al.*, 1997) (Fig. 1). Strain K22-20^T showed 97.7% 16S rRNA gene sequence similarity to *O. humiphilum* HKI 0124^T and low similarity values (95.0–89.0%) to members of the genera *Ornithinococcus*, *Demetria*, *Curto bacterium*, *Terracoccus*, *Promicromonospora*, *Beutenbergia*, *Kocuria*, *Dermatophilus*, *Sanguibacter*, *Cellulomonas*, *Stomatococcus*, *Arthrobacter*, *Bogoriella*, *Brachybacterium*, *Nesterenkononia*, *Rarobacter*, *Dermabacter*, *Microbacterium* and *Jonesia*.

To determine the genotypic relatedness between strain K22-20^T and *O. humiphilum* MTCC 6406^T, DNA–DNA hybridization studies were performed. The DNA–DNA

Table 2. Fatty acid compositions of strain K22-20^T and *O. humiphilum* MTCC 6406^T

Values are percentages of total fatty acids, determined in the present study. —, Not detected.

Fatty acid	K22-20 ^T	MTCC 6406 ^T
C _{15:0}	1.18	1.2
C _{16:0}	2.98	16.93
C _{17:0}	2.57	4.95
iso-C _{14:0}	0.66	—
iso-C _{15:0}	19.42	21.17
anteiso-C _{15:0}	6.52	1.72
iso-C _{16:0}	18.60	19.99
iso-C _{17:0}	12.40	7.73
iso-C _{17:1ω9c}	20.39	13.68
anteiso-C _{17:0}	7.92	3.1
anteiso-C _{17:1ω9c}	0.83	—
C _{17:1ω8c}	2.96	6.01
C _{17:0} 10-methyl	1.26	—
Summed feature 3*	0.79	7.83

*Summed feature 3 represents iso-C_{16:1ω7c} and/or C_{15:0} iso 2-OH, which could not be separated by GC with the MIDI Sherlock Identification system.

hybridization value was 64.5%, which is below the 70% cut-off value recommended for the delineation of bacterial species (Wayne *et al.*, 1987). The DNA G+C content of strain K22-20^T is 71 mol%. This polyphasic study revealed that strain K22-20^T could be clearly distinguished from the type species of the genus *Ornithinimicrobium*. Based on the above data, it is proposed that strain K22-20^T represents a novel species of the genus *Ornithinimicrobium*, *Ornithinimicrobium kibberense* sp. nov.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1488 bases) showing the phylogenetic relationship between strain K22-20^T and *Ornithinimicrobium humiphilum* and other members of the family *Intrasporangiaceae* and families of the suborder *Micrococcineae*. Bootstrap values greater than 500 (1000 replications) are given at the nodes. Bar, 1% sequence variation.

Description of *Ornithinimicrobium kibberense* sp. nov.

Ornithinimicrobium kibberense (kib.be.ren'se. N.L. neut. adj. *kibberense* pertaining to Kibber, a village in the Indian Himalayas, where the type strain was isolated).

Colonies on TSA are buff-yellow-pigmented and about 1–3 mm in diameter, circular, smooth, glistening, opaque and convex with an entire margin. Catalase-positive and oxidase-negative. Grows at 20–37 °C; optimum growth temperature is 28 °C and no growth occurs at 42 °C. NaCl is tolerated up to 7%. Positive for hydrolysis of starch and nitrate reduction and variable for casein hydrolysis. Negative for indole, hydrogen sulphide production and methyl red and Voges–Proskauer tests. Acid is produced from D-fructose, D-galactose, raffinose, sucrose, trehalose and D-maltose, but not from salicin, D-glucose, D-melibiose, L-arabinose, D-mannose, inulin, D-lactose, L-rhamnose, D-xylose, cellobiose or D-mannitol. Utilization of various substrates as sole carbon sources and sensitivity to antibiotics are given in Table 1. The diagnostic cell wall amino acid is L-ornithine and the interpeptide bridge consists of L-Orn, L-Ala, Gly and D-Asp. Whole-cell sugars are glucose, arabinose and xylose. The acyl type is acetyl. Major fatty acids are iso-C_{17:1}ω9c (20·39%), iso-C_{15:0} (19·42%), iso-C_{16:0} (18·60%) and iso-C_{17:0} (12·40%). The phospholipid is of type V, with phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and unknown glucosamine-containing phospholipids. The major menaquinone is MK-8(H₄). Mycolic acids are absent. The DNA G+C content of the type strain is 71 mol%.

The type strain is K22-20^T (=MTCC 6545^T=DSM 17687^T=JCM 12763^T), isolated from soil, 45 cm below an ice glacier, 4200 m above sea level, in Kibber village of the Spiti valley, Himachal Pradesh, India.

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