Shimazuella kribbensis gen. nov., sp. nov., a mesophilic representative of the family *Thermoactinomycetaceae*

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A mesophilic strain, designated A 9500^T, was isolated from a soil sample collected from Sobaek Mountain, South Korea, and its taxonomic position was investigated by using a polyphasic approach. The novel strain grew well on different ISP media and no diffusible pigments were produced. The optimum temperature for growth was 32 °C. The aerial mycelium was well developed, but not fragmented. The strain was Gram-positive, non-motile and formed endospores on vegetative and aerial hyphae with a spiny surface. Cell walls of strain A 9500^T contained meso-diaminopimelic acid as the diagnostic amino acid, but no characteristic sugars or other amino acids were found (chemotype-III). The major menaquinone was MK-9(H₄) and the minor menaquinone was MK-10(H₄) and they were detected at a ratio of 7:3. Phosphatidyl-ethanolamine was the diagnostic phospholipid. The G + C content of the genomic DNA was 39.4 mol%. The major fatty acids were anteiso-C_{15:0} (43.34 %), iso-C_{16:0} (14.23 %) and C_{16:0} (7.90 %), a composition that differed from members of related genera of the family *Thermoactinomycetaceae*. The novel strain formed a distinct clade in a phylogenetic tree based on 16S rRNA gene sequences. On the basis of a range of phenotypic and genotypic data and on 16S rRNA gene sequence similarities (88.35–90.38 %), it is suggested that strain A 9500^T represents a novel species in a new genus with the name *Shimazuella kribbensis* gen. nov., sp. nov. The type strain of the type species is A 9500^T (=KCTC 9933^T=DSM 45090^T).

The genus *Thermoactinomyces* (Tsilinsky, 1899) was the first described member of the family *Thermoactinomycetaceae* (Matsuo *et al.*, 2006). Recently, based on 16S rRNA gene sequence analysis, as well as chemotaxonomic and physiological characterization, the members of the genus *Thermoactinomyces* have been divided into six genera, *Thermoactinomyces sensu stricto* (Yoon *et al.*, 2005), *Laceyella* (Yoon *et al.*, 2005), *Thermoflavimicrobium* (Yoon *et al.*, 2005), *Seinonella* (Yoon *et al.*, 2005), *Planifilum* (Hatayama *et al.*, 2005) and *Mechercharimyces* (Matsuo *et al.*, 2006). At the time of writing, the reclassification of the family *Thermoactinomycetaceae* means that there are now the following recognized species: *Thermoactinomyces candidus* (Kurup *et al.*, 1975; proposed as a later synonym of *Thermoactinomyces vulgaris* by Yoon *et al.* 2000); *Thermoactinomyces vulgaris* (Tsilinsky, 1899); *Thermoactinomyces intermedius* (Kurup *et al.*, 1980);

Thermoactinomyces thalpophilus (Unsworth & Cross, 1980; proposed as a later synonym of *Thermoactinomyces sacchari* by Yoon *et al.* 2000); *Thermoflavimicrobium dichotomicus* (Krasil'nikov & Agre, 1964; Cross & Goodfellow, 1973); *Laceyella sacchari* (Lacey, 1971; Yoon *et al.*, 2005; basonym *Thermoactinomyces sacchari*); *Laceyella putida* (Lacey & Cross, 1989; Yoon *et al.*, 2005; basonym *Thermoactinomyces putidus*); *Seinonella peptonophila* (Nonomura & Ohara, 1971; Yoon *et al.*, 2005; basonym *Thermoactinomyces peptonophilus*); *Planifilum fimeticola* (Hatayama *et al.*, 2005); *Planifilum fulgidum* (Hatayama *et al.*, 2005); *Mechercharimyces mesophilus* (Matsuo *et al.*, 2006) and *Mechercharimyces asporophorigenens* (Matsuo *et al.*, 2006). These species are aerobic, Gram-positive and thermophilic, with the exception of two mesophilic species, *Seinonella peptonophila* and *Mechercharimyces mesophilus*. Here we describe another mesophilic member of the family *Thermoactinomycetaceae*.

Strain A 9500^T was isolated and maintained on Bennett's agar (Atlas, 1993) medium for 2 weeks at 30 °C. Other media used for cultural characteristics were inorganic

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salt–starch agar, yeast extract/malt extract agar, oatmeal agar, glycerol asparagine agar and tyrosine agar (Shirling & Gottlieb, 1966). The morphological characteristics of 7 day cultures of strain A 9500^T grown on Bennett's agar were observed by light microscopy (JEOL) and scanning electron microscopy (SEM 515; Philips). Morphological studies revealed that the new isolate shared the characteristics already described for the family *Thermoactinomycetaceae*, with white aerial mycelium and pale yellow substrate mycelium which was well developed. Substrate mycelium was branched, but not fragmented. Spores formed singly (1.0–1.4 × 0.7–0.9 µm) on aerial mycelium and were non-motile (Fig. 1a). Substrate hyphae were sessile and had extensively branched (0.3–0.6 µm) sporophores with the structure and properties of bacterial endospores (Fig. 1b). The colours of the substrate and aerial mycelia and any soluble pigments were determined (Kelly, 1964). Strain A 9500^T grew on all media tested. Good growth was observed on Bennett's agar and no diffusible pigments were produced on the media tested.

The temperature range for growth was 20–50 °C, with the optimum temperature at 32 °C. The pH and NaCl tolerance was examined as described by Tang *et al.* (2003). Other physiological features and carbon source utilization were observed on a basal medium used for cultivation of members of the genus *Thermoactinomyces* (Williams *et al.*, 1989). Detailed physiological and biochemical characteristics of the novel isolate are given in Table 1 and in the species description.

Fig. 1. Scanning electron micrographs of cells of *Shimazuella kribbensis* sp. nov. A 9500^T grown on ISP medium 2 for 7 days at 32 °C, showing aerial mycelium spores (a) and spiny spores on the substrate mycelium (b). Bars, 1 µm (a) and 10 µm (b).

The chemotaxonomic characteristics of strain A 9500^T were determined using cells cultured in Bennett's medium at the exponential phase of growth. The composition of the amino acids and sugars in the cell-wall peptidoglycan was determined by the methods of Lechevalier & Lechevalier (1980) and Schleifer & Kandler (1972) using cellulose TLC plates. The menaquinone and cellular fatty acid profiles were determined as described by Collins *et al.* (1977) and were analysed by HPLC (Kroppenstedt, 1982). The cell-wall peptidoglycan of the novel isolate contained *meso*-diaminopimelic acid (*meso*-DAP), glutamic acid and alanine, but no diagnostic sugars. Thus strain A 9500^T had an unusual type III cell wall (Lechevalier & Lechevalier, 1970). This result was consistent with that obtained previously for other members of the family *Thermoactinomycetaceae*. The diagnostic phospholipid was phosphatidylethanolamine. However, the predominant menaquinones were MK-9(H₄) and MK-10(H₄) in a ratio of 7:3. For the above analysis, the organism was cultivated on Bennett's broth for 3 days at 32 °C and harvested by centrifugation at 3000 r.p.m. for 20 min. Cellular fatty acid analysis was performed as described by Sasser (1990) using the Microbial Identification System (MIDI). The fatty acid profile of strain A 9500^T was mainly composed of anteiso-C_{15:0} (43.34 %), iso-C_{16:0} (14.23 %), C_{16:0} (7.90 %), iso-C_{15:0} (7.40 %), anteiso-C_{17:0} (7.17 %), iso-C_{14:0} (6.14 %), C_{15:0} (4.01 %) and anteiso-C_{16:0} (3.76). The G + C DNA content of strain A 9500^T was determined by using the HPLC method of Mesbah *et al.* (1989) and was 39.4 mol%.

Extraction and amplification of genomic DNA for 16S rRNA gene sequence analysis was carried out as described by Cui *et al.* (2001). The 16S rRNA gene fragment was amplified by using universal primers corresponding to positions 8–27 for the forward primer and 1492–1510 for the reverse primer (*Escherichia coli* numbering system; Weisburg *et al.*, 1991). Based on 1459 bp long 16S rRNA gene sequences, phylogenetically related bacteria were aligned by using a BLAST search (Altschul *et al.*, 1990) against the GenBank database. Multiple alignments with sequences of related taxa of the family *Thermoactinomycetaceae* were implemented by using CLUSTAL_X (Thompson *et al.*, 1997). The 16S rRNA gene sequence similarity values were calculated by pairwise comparison (Kimura, 1980). Phylogenetic trees (Fig. 2) were constructed based on different tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), least squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Fitch, 1971) methods. A neighbour-joining tree was reconstructed from evolutionary distances calculated using the Jukes–Cantor coefficient (Jukes & Cantor, 1969). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

In the phylogenetic dendrogram, strain A 9500^T formed a stable clade (supported by a 63 % bootstrap value, see Fig. 2) with *Laceyella putida* KCTC 3666^T. The highest 16S

Fig. 2. Phylogenetic dendrogram based on 16S rRNA gene sequence analysis, reconstructed from evolutionary distances by using the neighbour-joining method, showing the position of strain A 9500^T within the family *Thermoactinomycetaceae*. Asterisks indicate branches that were recovered using least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Fitch, 1971) algorithms. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per nucleotide position. *Bacillus subtilis* NCDO 1769^T was used as the outgroup.

rRNA gene similarity values found between strain A 9500^T and other genera of the family *Thermoactinomycetaceae* were with *Thermoactinomyces vulgaris* KCTC 9557 (90.38 % gene sequence similarity), *Thermoflavimicrobium dichotomicum* KCTC 3667^T (90.22 %), *Laceyella sacchari* KCTC 9790^T (90.04 %), *Thermoactinomyces intermedius* KCTC 9646^T (89.98 %), *Laceyella putida* KCTC 3666^T (89.67 %), *Mechercharimyces asporophorigenens* DSM 44955^T (89.50 %), *Mechercharimyces mesophilus* DSM 44894^T (89.41 %), *Seinonella peptonophila* KCTC 9740^T

(89.41 %), *Planifilum fimeticola* JCM 12507^T (88.43 %) and *Planifilum fulgidum* JCM 12508^T (88.35 %). The length of sequence compared was 1459 bp.

It is evident from the gene sequence similarity values, the phylogenetic tree (Fig. 2) and the phenotypic properties (Table 1) that strain A 9500^T can be clearly distinguished from other genera of the family *Thermoactinomycetaceae*. Examination of the growth temperature, DNA G+C contents, menaquinone composition, cellular fatty acid

Table 1. Phenotypic differentiation of strain A 9500^T and related genera of the family *Thermoactinomycetaceae*

Taxa: 1, strain A 9500^T; 2, *Laceyella*; 3, *Mechercharimyces*; 4, *Planifilum*; 5, *Seinonella*; 6, *Thermoactinomyces*; 7, *Thermoflavimicrobium*. +, Positive reaction; –, negative reaction; +/–, variable reaction; NO, not observed; ND, no data. Data from this study, Yoon *et al.* (2005), Hatayama *et al.* (2005) and Matsuo *et al.* (2006).

Characteristic	1	2	3	4	5	6	7
Colour of aerial mycelia	White	White	White	NO	White	White	Yellow
Growth on 25 µg novobiocin ml ⁻¹	+	+	+	ND	–	+	+
Degradation of:							
Casein	+	+	+	+	–	+	+
Gelatin	–	+	+	ND	–	+	+
Starch	+	–	+	+	–	+	+
Hypoxanthine	–	–	–	–	–	–	+
Xanthine	–	–	–	–	–	–	+
L-Tyrosine	–	+ / –	–	–	–	–	–
Optimum temperature for growth (°C)	32	48–55	30	55–63	35	50–55	55
Predominant menaquinone	MK-9	MK-9	MK-9	MK-7	MK-7	MK-7	MK-7
Other menaquinone(s)	MK-10	MK-7, MK-8 or MK-10	MK-8	ND*	MK-8, MK-9, MK-10	MK-8 or MK-9	ND*
Major fatty acids	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0} , C _{16:0} , anteiso-C _{17:0}	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{15:0} , iso-C _{17:0} ω11c	iso-C _{17:0} , anteiso-C _{17:0} , (iso-C _{15:0} or C _{16:0})	iso-C _{14:0} , anteiso-C _{15:0} , iso-C _{16:0}	iso-C _{15:0} , iso-C _{17:0} , anteiso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0}
DNA G+C content (mol%)	39.4	48.0–49.0	45.0	58.7–60.3	40.0	48.0	43.0

*MK-8 detected at a trace level of 1.9 % peak area ratio (Hatayama *et al.*, 2005).

content and the ability to degrade gelatin reveals that strain A 9500^T does not belong to the genera *Thermoactinomyces sensu stricto*, *Laceyella*, *Thermoflavimicrobium*, *Seinonella*, *Planifilum* or *Mechercharimyces*. On the basis of the results presented in this study, strain A 9500^T has been assigned to a novel genus in the family *Thermoactinomycetaceae*, for which the name *Shimazuella kribbensis* gen. nov., sp. nov. is proposed.

Description of *Shimazuella* gen. nov.

Shimazuella (Shi.ma.zu.el'la. N.L. fem. n. *Shimazuella* named after Akira Shimazu, a Japanese microbiologist from Tokyo University, who has contributed to the field of prokaryotic taxonomy).

Cells are Gram-positive, aerobic and mesophilic. Aerial mycelium is abundant and white and is not fragmented. Forms well-developed, branched and septate substrate mycelia on Bennett's agar and yeast extract-malt extract (ISP 2). Soluble pigments are not produced. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid, glutamic acid and alanine, but no characteristic sugars. Endospores are produced singly along mycelia. The major menaquinone is MK-9 and is found at a ratio of 7:3 with MK-10. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0}, C_{16:0}, iso-C_{15:0} and anteiso-C_{17:0}. The G+C content is 39.4 mol%. The type species is *Shimazuella kribbensis*.

Description of *Shimazuella kribbensis* sp. nov.

Shimazuella kribbensis (krib.ben'sis. N.L. fem. adj. *kribbensis* pertaining to KRIBB, an arbitrary adjective formed from the acronym of the Korea Research Institute of Bioscience and Biotechnology, KRIBB, where the taxonomic studies on this new genus and novel species were performed).

In addition to the genus description, exhibits the following characteristics. Colonies are fast-growing, ridged with white mycelia and a feathery margin on Bennett's agar at 28 °C. Growth occurs between 20 and 37 °C, with optimum growth at 32 °C. Forms endospores singly on unbranched sporophores. Casein and starch are degraded, but not gelatin, hypoxanthine, xanthine or L-tyrosine. No pigments are observed on all media tested. Growth occurs in the presence of 25 µg novobiocin ml⁻¹. Major cellular fatty acids are anteiso-C_{15:0}, iso-C_{16:0}, C_{16:0}, iso-C_{15:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 39.4 mol%.

The type strain, A 9500^T (=KCTC 9933^T=DSM 45090^T), was isolated from a soil sample collected from Sobaek mountain, Republic of South Korea.

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