Kroppenstedtia eburnea gen. nov., sp. nov., a thermoactinomycete isolated by environmental screening, and emended description of the family *Thermoactinomycetaceae* Matsuo *et al.* 2006 emend. Yassin *et al.* 2009

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A Gram-positive, spore-forming, aerobic, filamentous bacterium, strain JFMB-ATE^T, was isolated in 2008 during environmental screening of a plastic surface in grade C in a contract manufacturing organization in southern Germany. The isolate grew at temperatures of 25–50 °C and at pH 5.0–8.5, forming ivory-coloured colonies with sparse white aerial mycelia. Chemotaxonomic and molecular characteristics of the isolate matched those described for members of the family *Thermoactinomycetaceae*, except that the cell-wall peptidoglycan contained LL-diaminopimelic acid, while all previously described members of this family display this diagnostic diamino acid in *meso*-conformation. The DNA G + C content of the novel strain was 54.6 mol%, the main polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol, and the major menaquinone was MK-7. The major fatty acids had saturated C₁₄–C₁₆ branched chains. No diagnostic sugars were detected. Based on the chemotaxonomic results and 16S rRNA gene sequence analysis, the isolate is proposed to represent a novel genus and species, *Kroppenstedtia eburnea* gen. nov. sp. nov. The type strain is JFMB-ATE^T (=DSM 45196^T =NRRL B-24804^T =CCUG 59226^T).

Based on morphological characteristics, the filamentous, Gram-positive species of the genus *Thermoactinomyces* were initially considered to belong to the order *Actinomycetales*, before they were placed within the distinct family *Bacillaceae*, primarily due to phylogenetic studies of the 16S rRNA gene (Stackebrandt & Woese, 1981), but in agreement with phenotypic features such as DNA G + C content and endospore formation (Cross *et al.* 1968). Later, the members of the genus *Thermoactinomyces* were divided into the genera *Thermoactinomyces*, *Laceyella*, *Thermoflavimicrobium* and *Seinonella* (Yoon *et al.* 2005), corresponding to distinct phylogenetic groups with respective phenotypic characters, such as unique profiles of menaquinone and cellular fatty acid compositions. The

genus *Planifilum* (Hatayama *et al.* 2005) joined this group, which was subsequently reunited by the introduction of the family *Thermoactinomycetaceae* (within the order *Bacillales*), together with the genus *Mechercharimyces* (Matsuo *et al.* 2006). More recently, the genera *Shimazuella* (Park *et al.* 2007) and *Desmospora* (Yassin *et al.* 2009) were included in the family *Thermoactinomycetaceae*. Most species of this family are thermophilic, as indicated by the family name [Gr. adj. *thermos* hot; Gr. n. *aktis* -inos ray, beam; Gr. masc. n. *mukês* a mushroom, anything shaped like a mushroom; N.L. masc. n. *Thermoactinomycetes* heat (loving) ray fungus], although several of the member genera, such as *Seinonella*, *Mechercharimyces* and *Shimazuella*, are mesophilic. Strains of the family *Thermoactinomycetaceae* have been isolated from different sources such as mouldy hay, cereal grains, soil, mushroom compost, river water, marine sediments, dairy products, humidifiers of air-conditioning systems, sputa from a patient with suspected pulmonary tuberculosis and other clinical and environmental sources.

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JFMB-ATE^T is FN665656.

Strain JFMB-ATE^T was isolated during environmental screening of a plastic surface in grade C in a contract manufacturing organization in southern Germany, using TSA (tryptic soy agar; tryptic soy from Becton Dickinson) medium at a temperature between 30 and 35 °C. The organism was cultivated on Brain Heart Infusion agar (BHI; Becton Dickinson), initially at 28 °C and subsequently at 40 °C. For the determination of morphological characteristics according to Shirling & Gottlieb (1966), strain JFMB-ATE^T was cultivated on the four suggested media (yeast extract–malt extract agar, oatmeal agar, inorganic salts–starch agar and glycerol–asparagine agar), together with cultivation on media from DSMZ (List of growth media; http://www.dsmz.de/microorganisms/media_list.php): GPHF medium (DSMZ-medium 553), trypticase soy broth agar (535) and GYM *Streptomyces* medium (65). Electron micrographs were taken with a scanning electron microscope (DSM 982 Gemini; Zeiss) and light microscopic images were made with $\times 400$ magnification and phase-contrast illumination using a Zeiss Axio Scope A1. In both cases, mycelia of strain JFMB-ATE^T, which were grown for 3 days in liquid BHI medium at 45 °C, were examined. Growth rates were determined at temperatures from 25 to 60 °C and at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Production of endospores was tested by exposure of the cells to 98 °C for distinct intervals (10 min, 30 min, 1 h, 2 h, 4 h) followed by transfer to agar plates at 45 °C and evaluation for viable cells after a period of 2 days. The production of melanoid pigments was investigated by cultivation of strain JFMB-ATE^T on tyrosine agar (DSMZ medium 5011; L-tyrosine from Merck) and peptone–iron agar (DSMZ medium 5010; peptone from Merck). Degradation of specific substrates was examined using agar plates with various basal media. Casein degradation was tested on plates containing milk powder (5%, w/v), NaCl (0.5%) and agarose (1.0%); positive results are indicated by clear zones around the colonies. Gelatin degradation was examined on plates containing nutrient broth (0.8%), gelatin (0.4%) and agarose (1.5%). After growth, plates were covered with 0.5 M H₂SO₄, saturated with Na₂SO₄ solution at 55 °C; positive results are indicated by clear zones around the colonies. The decomposition of xanthine was tested as described by Gordon & Mihm (1957) and that of hypoxanthine by the same test, replacing xanthine by the same amount of hypoxanthine. Tyrosine degradation was investigated on plates containing glycerol (1.5%), L-tyrosine (0.05%), L-asparagine (0.1%), K₂HPO₄ (0.05%), MgSO₄·7H₂O (0.05%), NaCl (0.05%), trace salt solution (0.1%, v/v), CuSO₄ (0.001%) and agarose (1.5%); positive results are indicated by the production of a dark brown pigment around the colonies. Starch degradation was tested on plates containing nutrient broth (0.8%), starch (1%) and agarose (1.5%), with a positive result indicated by clear zones around the colonies. Chitin degradation was investigated on plates containing bleached chitin powder (0.4%), K₂HPO₄ (0.07%), KH₂PO₄ (0.03%), MgSO₄·7H₂O (0.05%), FeSO₄·7H₂O (0.001%), ZnSO₄·7H₂O (0.0001%), MnCl₂·4H₂O (0.0001%) and agarose (1.2%); positive results are indicated by clear zones around the colonies. Aesculin degradation was examined in soft-agar tubes

containing yeast extract (0.3%), aesculin (0.1%), ammonium iron (III) citrate (0.05%) and agarose (0.3%); positive results are indicated by the disappearance of the fluorescence caused by the aesculin in the medium. Furthermore, the use of D-glucose, L-arabinose, sucrose, D-xylose, i-inositol, D-mannitol, D-fructose, rhamnose and raffinose as carbon sources was examined, using carbon utilization medium (Shirling & Gottlieb, 1966), and the utilization of L-alanine, L-arginine, L-asparagine, L-cysteine, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-proline, L-serine, L-threonine, L-tryptophan and L-valine (all 0.1%, w/v) as nitrogen sources was assessed in a basal medium consisting of D-glucose (1%, w/v), MgSO₄·7H₂O (0.05%), NaCl (0.05%), FeSO₄·7H₂O (0.001%), K₂HPO₄ (0.1%) and Bacto-agar (Difco) (1.2%), at pH 7.4. Further biochemical tests were performed as described by Meganathan *et al.* (2007) and Tindall *et al.* (2007), including alcohol dehydrogenase assay 22.7.4, indole production (15.2.39), Voges–Proskauer reaction (15.2.82), urease activity (15.2.81) and nitrate reduction/denitrification (15.2.57). Whole cell amino acids and sugars were prepared according to Lechevalier & Lechevalier (1970) and analysed by thin layer chromatography (Staneck & Roberts, 1974). Phospholipids were extracted, separated by two-dimensional thin layer chromatography and identified according to Minnikin *et al.* (1984) as modified by Kroppenstedt & Goodfellow (2006). For the extraction of menaquinones, freeze-dried cell material was extracted with methanol as described by Collins *et al.* (1977) and analysed by HPLC (Kroppenstedt, 1982). Cellular fatty acids were analysed using the Microbial Identification System (MIDI) Sherlock Version 4.5, database ACTIN1, and by following described methods (Sasser, 1990). Peptidoglycan preparations purified according to the method of Schleifer (1985) were obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion. The amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending thin-layer chromatography on cellulose plates by using previously described solvent systems (Schleifer, 1985). The molar ratios of amino acids were determined by gas chromatography and gas chromatography-mass spectrometry of *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987; Groth *et al.*, 1996). The G + C content of the chromosomal DNA was determined by HPLC according to Mesbah *et al.* (1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out as described by Rainey *et al.* (1996). Phylogenetic trees were inferred by maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) algorithms, using the PHYLIP software package (Felsenstein, 1993). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings using both the maximum-likelihood and neighbour-joining algorithms. The root position was estimated by using sequences of *Aneurinibacillus thermoaerophilus* DSM 10154^T and *Bacillus fortis* DSM 16012^T as outgroups.

Strain JFMB-ATE^T exhibited fast, abundant growth on BHI medium, producing cloudy, ivory coloured substrate mycelia (Figs 1 and 2). The white aerial mycelia were sparse. Colonies were flat, of irregular shape with undulate margin and had a dull surface which later formed radial wrinkles. Growth was also observed on GYM *Streptomyces* medium, while no growth occurred on yeast extract–malt extract agar, oatmeal agar, inorganic salts–starch agar, glycerol–asparagine agar, GPHF medium and trypticase soy broth agar, reflecting the fact that most *Streptomyces* media are not rich enough for the cultivation of species of the family *Thermoactinomycetaceae*. Microscopic examination revealed long, straight to flexuous, moderately branched septate hyphae, developing single endospores on unbranched sporophores and chains of arthrospores in a zigzag arrangement as previously reported for *Desmospora activa* (Yassin *et al.* 2009). Colonies of strain JFMB-ATE^T produced no diffusible pigments on BHI or GYM medium. The investigation of melanoid pigment production by cultivation on tyrosine agar and peptone–iron agar revealed that no melanoid pigments were produced. The organism grew at temperatures between 25 and 50 °C, with an optimum at 45 °C. Abundant growth was observed at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5; the pH variation did not detectably influence growth rate. The spore test indicated that endospores are produced, as colonies formed after exposure to 98 °C for as long as 4 h. Casein, gelatin and aesculin were hydrolysed, while xanthine, hypoxanthine, L-tyrosine, starch and chitin were not. The chemotaxonomic properties of strain JFMB-ATE^T are compared with those of other genera within the family *Thermoactinomycetaceae* in Table 1. The tests for carbon source utilization showed abundant growth on sucrose, i-inositol, D-mannitol and rhamnose, and weak growth on L-arabinose, D-xylose, D-fructose and raffinose, while on D-glucose, no difference from the negative control was observed. The nitrogen utilization assay revealed no growth on any of the given substrates, likely indicating that the basal medium used is not rich enough for examining species of the family *Thermoactinomycetaceae*. Further metabolic tests revealed a positive result for urease activity and negative results for alcohol dehydrogenase, indole production, Voges–Proskauer reaction and nitrate reduction/denitrification. Analysis of cell-wall components revealed the presence of LL-diaminopimelic acid (DAP), which is a unique feature of strain JFMB-ATE^T among all presently described species of the family *Thermoactinomycetaceae*, and at variance with the description of the family (Matsuo *et al.*, 2006; Yassin *et al.*, 2009). The hydrolysate (4 M HCl, 100 °C, 16 h) of the peptidoglycan preparation of strain DSM 45196^T contained LL-DAP, alanine (Ala), glycine (Gly) and glutamic acid (Glu) in a molar ratio of 1.2:0.9:0.4:1.0. The partial hydrolysate (4 M HCl, 100 °C, 0.75 h) contained in addition to these amino acids the peptides L-Ala–D-Glu and L-Lys–D-Ala. From these data it was concluded that strain DSM 45196^T shows the peptidoglycan type A3γ LL-DAP–Gly (Schleifer & Kandler 1972; type A41.1 according to <http://www.dsmz>.

Fig. 1. Phase-contrast microscopic images showing sterile substrate mycelium of strain JFMB-ATE^T, which was cultivated for 2 days in BHI medium at 40 °C. Arrows in (a) mark nascent endospores, in (b) mature endospores formed singly on unbranched sporophores. Bars, 10 μm.

Fig. 2. Scanning electron micrograph showing sterile substrate mycelium of strain JFMB-ATE^T, which was cultivated for 2 days in BHI medium at 40 °C. Arrows indicate positions of constriction, likely due to initiating fragmentation, representing an early stage of arthrospore formation. Bar, 1 μm.

Table 1. Phenotypic and molecular genetic characteristics of strain JFMB-ATE^T and related genera of the family *Thermoactinomycetaceae*

Taxa: 1, strain JFMB-ATE^T (data from this study); 2, *Desmospora* (data from Yassin *et al.* 2009); 3, *Mechercharimyces* (Matsuo *et al.*, 2006); 4, *Thermoactinomyces* (Lacey & Cross, 1989; Yoon *et al.*, 2005); 5, *Laceyella* (Lacey & Cross, 1989; Yoon *et al.*, 2005); 6, *Thermoflavimicrobium* (Lacey & Cross, 1989; Yoon *et al.*, 2005); 7, *Planifilum* (Hatayama *et al.*, 2005; Zhang *et al.*, 2007); 8, *Seinonella* (Yoon *et al.*, 2005); 9, *Shimazuella* (Park *et al.*, 2007). +, Positive reaction; –, negative reaction; +/–, variable reaction; ND, no data available; NO, not observed.

Characteristic	1	2	3	4	5	6	7	8	9
Major menaquinone	MK-7	MK-7	MK-9	MK-7	MK-9	MK-7	MK-7	MK-7	MK-9
Further detected menaquinones	NO	NO*	MK-8	MK-8 or MK-9	MK-7, MK-8 or MK-10	NO*	NO	MK-8, MK-9, MK-10	MK-10
Major fatty acids†	i-C _{15:0} , ai-C _{15:0}	i-C _{15:0} , i-C _{17:0} , C _{16:0}	i-C _{15:0} , i-C _{17:1} ω11c	i-C _{15:0} , i-C _{17:0} , ai-C _{15:0}	i-C _{15:0} , ai-C _{15:0}	i-C _{15:0} , ai-C _{15:0} , i-C _{16:0}	i-C _{17:0} , ai-C _{17:0} , (i-C _{15:0} , C _{16:0} or i-C _{16:0})	i-C _{14:0} , ai-C _{15:0} , i-C _{16:0}	i-C _{15:0} , ai-C _{15:0} , i-C _{16:0} , C _{16:0} , ai-C _{17:0}
Diaminopimelic acid	LL-DAP	meso-DAP	meso-DAP	meso-DAP	meso-DAP	meso-DAP	meso-DAP	meso-DAP	meso-DAP
DNA G + C content (mol%)	54.6	49.3	45.0	48.0	48.0–49.0	43.0	56.8–60.3	40.0	39.4
Colour of aerial mycelium	White	Yellow	White	White	White	Yellow	NO	White	White
Growth with 25 µg novobiocin ml ⁻¹	+	+	+	+	+	+	+	–	+
Degradation of:									
Casein	+	+	+	+	+	+	+	–	+
Aesculin	+	+	–	+	+/–	–	+	ND	–*
Gelatin	+	+	+	+	+	+	+	–	–
Starch	–	+	–	–	+	–	+	–	+
Hypoxanthine	–	–	–	–	–	+	–	–	–
Xanthine	–	–	–	–	–	+	–	–	–
L-Tyrosine	–	–	–	+/–	+/–	–	+/–	–	–
Optimal temperature for growth (°C)	45	30–50	30	50–55	48–55	55	55–70	35	32

*Data from this study, based on experiments with the type strains of *D. activa*, *T. dichotomicum*, *P. fulgidum*, *P. fimeticola* and *S. kribbensis*.

†Only components making up >10% peak area ratio are shown.

de/microorganisms/main.php?content_id=35). Moreover, strain JFMB-ATE^T displayed only menaquinone MK-7. Major fatty acids were saturated branched-chain acids: iso-C_{15:0} (73.3%), anteiso-C_{15:0} (13.1%), iso-C_{16:0} (4.5%) and iso-C_{14:0} (3.9%). The phospholipid pattern consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and unknown phospholipids, typical of phospholipid pattern type PII *sensu* Lechevalier *et al.* 1977. Whole-cell sugar analysis revealed only the presence of glucose and ribose; no diagnostic sugars were detected (Lechevalier & Lechevalier, 1970). The G + C content of the chromosomal DNA was 54.6 mol%. Previously unreported properties of genera of the family *Thermoactinomycetaceae* were determined in this study (see Table 1), based on experiments with *D. activa* (DSM 45169^T), *Thermoflavimicrobium dichotomicum* (DSM 44778^T), *Planifilum fulgidum* (DSM 44945^T), *Planifilum fimeticola* (DSM 44946^T) and *Shimazuella kribbensis* (DSM 45090^T). Results for the degradation of aesculin by members of the genus *Planifilum* were only obtained from *P. fimeticola*, as growth of *P. fulgidum* was not observed in our analyses.

Using EzTaxon (Chun *et al.*, 2007) for comparison of the 16S rRNA gene sequence of strain JFMB-ATE^T with those of the type species within the family *Thermoactinomycetaceae* with validly published names revealed the highest degree of sequence similarity with *P. fimeticola* (93.1%), *D. activa* (93.0%), *Planifilum yunnanense* (93.0%), *P. fulgidum* (92.9%), *Mechercharimyces mesophilus* (92.1%), *Laceyella putida* (91.9%), *Mechercharimyces asporophorigenens* (91.8%), *Laceyella sacchari* (91.7%), *Thermoactinomycetes intermedius* (91.3%), *Thermoflavimicrobium dichotomicum* (91.0%) and *Thermoactinomycetes vulgaris* (91.0%). Both the maximum-likelihood and neighbour-joining phylogenetic trees demonstrate that strain JFMB-ATE^T clusters nearest to the genus *Planifilum*; however, the high degree of 16S rRNA gene sequence dissimilarity ($\geq 6.9\%$) indicates only a distant relationship.

Several phenotypic characteristics, apart from the phylogenetic analysis based on 16S rRNA gene sequences, also support the distinctiveness of strain JFMB-ATE^T from all other described members of the family *Thermoactinomycetaceae*

(see Table 1). In particular, a unique feature of this strain is the presence of LL-DAP in the cell-wall peptidoglycan rather than the *meso* isomer which is present in all other members of this family. Moreover, strain JFMB-ATE^T differs from the majority of taxa within the family *Thermoactinomycetaceae*, with the exception of the genus *Planifilum*, by having a significantly higher DNA G + C content. The topology of the phylogenetic tree (Fig. 3), where strain JFMB-ATE^T clusters with the genus *Planifilum*, possibly suggests that the tendency towards high G + C DNA composition is a specific development of the common branch containing the genus *Planifilum* and strain JFMB-ATE^T. However, the latter is clearly distinct from the genus *Planifilum*, not only by 16S rRNA gene sequence divergence and the presence of LL-DAP in the peptidoglycan, but also by iso-C_{15:0} and anteiso-C_{15:0} as the major fatty acids versus iso-C_{17:0} and anteiso-C_{17:0} as the dominant fatty acids in members of the genus *Planifilum*. Likewise, the fatty acid composition also differentiates strain JFMB-ATE^T from the members of the genus *Seinonella*, while the major menaquinone differentiates it from the genera *Mechercharimyces*, *Laceyella* and *Shimazuella*. Finally, strain JFMB-ATE^T further differs from members of the genus *Shimazuella* by the ability to hydrolyse gelatin and by the inability to hydrolyse starch.

Description of the genus *Kroppenstedtia* gen. nov.

Kroppenstedtia (Krop.pen.sted'ti.a. N.L. fem. n. *Kroppenstedtia* named in honour of Reiner M. Kroppenstedt, a German microbiologist, who has contributed significantly to the field of bacterial taxonomy).

Cells are aerobic, Gram-positive and chemoorganotrophic. Thermotolerant growth occurs between 25 and 50 °C, and at pH 5.0–8.5. Substrate and aerial mycelia are formed, both producing chains of arthrospores and heat-resistant endospores, the latter are formed singly on unbranched sporophores. The predominant menaquinone is MK-7. Major fatty acids are iso-C_{15:0} and anteiso-C_{15:0}. The cell wall peptidoglycan contains LL-DAP and is of type A3 γ LL-DAP–Gly (Schleifer & Kandler, 1972). Diagnostic sugars

Fig. 3. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain JFMB-ATE^T in relation to type strains of the family *Thermoactinomycetaceae*. Bar, 0.01 substitutions per nucleotide position. Bootstrap values greater than 50% are shown at branch points; values above branches refer to calculations based on the maximum-likelihood algorithm; values below branches are based on neighbour-joining.

are not detected in whole-cell hydrolysates, implying cell-wall chemotype I *sensu* Lechevalier & Lechevalier, 1970. The phospholipid pattern predominantly consists of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol, indicating phospholipid pattern type PII *sensu* (Lechevalier *et al.*, 1977). The DNA G+C content of the type strain of the type species is 54.6 mol%. The type species is *Kroppenstedtia eburnea*.

Description of *Kroppenstedtia eburnea* sp. nov.

Kroppenstedtia eburnea (e.bur'ne.a. L. fem. adj. *eburnea* of ivory, referring to the colony colour of the type strain).

Colonies are ivory coloured, cloudy, flat and of irregular shape with undulate margin and a dull surface, forming radial wrinkles and sparse white aerial mycelia. No diffusible pigments are produced on BHI and GYM media and no melanoid pigments on peptone–iron agar. Casein, gelatin and aesculin are hydrolysed; starch, xanthine, hypoxanthine, chitin and L-tyrosine are not hydrolysed. Urease reaction is positive. Alcohol dehydrogenase, indole production, nitrate reduction/denitrification and Voges–Proskauer reaction are negative.

The type strain, JFMB-ATE^T (=DSM 45196^T =NRRL B-24804^T =CCUG 59226^T) was isolated during environmental screening of a plastic surface in grade C in a contract manufacturing organization in southern Germany.

Emended description of the family *Thermoactinomycetaceae* Matsuo *et al.* 2006 emend. Yassin *et al.* 2009

Thermoactinomycetaceae (Ther'mo.ac.ti.no.my.ce.ta'ce.ae. N.L. masc. n. *Thermoactinomyces* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Thermoactinomycetaceae* the *Thermoactinomyces* family).

The description of the family *Thermoactinomycetaceae* is as given by Yassin *et al.* (2009), with the following amendment: cell walls contain *meso*-diaminopimelic acid (*meso*-DAP) or LL-diaminopimelic acid (LL-DAP).

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