***Thermobifida cellulolytica* sp. nov., a novel lignocellulose-decomposing actinomycete**

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Four actinomycete strains, isolated from the overheated region of manure compost, were assigned to the genus *Thermobifida* on the basis of morphological, physiological and biochemical characteristics. All strains produced single, ovoid, heat-sensitive spores on dichotomically branched aerial hyphae. On the basis of chemotaxonomic traits, these isolates showed strong affinity towards members of the genus *Thermobifida*. Cell-wall analysis revealed the presence of meso-diaminopimelic acid, but no other characteristic amino acids or sugars in the murein (cell wall type III). According to polar lipid analysis, all strains showed PL II-type phospholipid composition; phosphatidylethanolamine and glycolipid were detected together with some unidentified phospholipids. The isoprenoid quinone composition of the new isolates differed slightly from that of the other two *Thermobifida* species described thus far. The partial 16S rDNA sequence similarity of the four strains reached 99.8–100%, whereas a nearly complete 16S rDNA sequence of TB100^T, the representative strain of this collection, showed only 97.4 and 97.8% similarity to the corresponding rDNA sequences of the type strains of *Thermobifida fusca* and *Thermobifida alba*, respectively. These four isolates constituted a homogeneous group with levels of DNA–DNA homology ranging from 94.6 to 99.1%. The DNA–DNA relative homology values of strain TB100^T to *Thermobifida fusca* ATCC 27730^T and *Thermobifida alba* DSM 43795^T were 48.1 and 57%, respectively. On the basis of phenotypic, chemotaxonomic and genotypic data, the strains are assigned to a new species within the genus *Thermobifida* under the name *Thermobifida cellulolytica* sp. nov. The type strain is TB100^T (= DSM 44535^T = NCAIM B01997^T).

Keywords: *Thermomonospora*, *Thermobifida*, cellulolytic actinomycetes, *Thermobifida cellulolytica* sp. nov.

INTRODUCTION

The genus *Thermobifida* was constructed by Zhang *et al.* (1998) for actinomycetes assigned originally to the highly heterogeneous genus *Thermomonospora*. Six species of *Thermomonospora*, including *Thermomonospora fusca* and *Thermomonospora alba*, have formerly

been accepted and listed in the 9th edition of *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994). Phylogenetic analysis, based on 16S rRNA sequences, resulted in a reclassification of this group of actinomycetes. *Thermomonospora curvata* was the only species left in the genus *Thermomonospora*, *Thermomonospora mesophila* was transferred into *Microbispora*, and *Thermomonospora formosensis* was moved to the genus *Actinomadura*, whereas *Thermobifida fusca* and *Thermobifida alba* were affiliated into a new genus, *Thermobifida* gen. nov., belonging to the family *Nocardiopsaceae*; *Thermobifida alba* was designated as the type species. The taxonomic position of *Thermomonospora chromogena* remained uncertain.

Stereoscopic images of spore clusters of *Thermobifida cellulolytica* are available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>).

The GenBank/EMBL/DBJ accession numbers for partial 16S rRNA gene sequences are AJ298058, AJ298061, AJ29060 and AJ298059 for strains TB100^T, TB110, TB108 and K21, respectively.

Members of the genus *Thermobifida* are Gram-positive, non acid-fast, chemo-organotrophic aerobic organisms that form an extensively branched substrate mycelium. They produce single, heat-sensitive spores borne on dichotomously branched sporophores forming dense spore clusters. Their cell walls contain *meso*-diaminopimelic acid (wall chemotype III), they are rich in fatty acids of the iso and anteiso series (fatty acid type 3e) and contain MK-10(H₆), MK-10(H₈) and MK-11(H₆) menaquinones (menaquinone type 4D); the major polar lipids are phosphatidylethanolamine and glycolipid (the phospholipid pattern is type II). The habitats are soil, manures and overheated compost. The outstanding cellulose-decomposing capability is the major characteristic feature of strains assigned to this two-membered new genus.

Searches for lignocellulolytic actinomycetes from the hot core of manure compost yielded the isolation of four strains (K21, TB100^T, TB108, TB110) with morphological structures typical of the genus *Thermobifida*. All strains showed high levels of cellulase (including cellobiohydrolase and endoglucanase), endoxylanase, mannosidase, protease and amylase activity, as well as lignin-solubilization activity (Kukolya *et al.*, 1997).

In this paper, we report the morphological, physiological, chemotaxonomical and genetic characteristics of these isolates. The detailed description of a new species, *Thermobifida cellulolytica* sp. nov., is also presented.

METHODS

Strains and culture conditions. The strains used in this study were K21, TB100^T, TB108 and TB110, isolated as described earlier (Kukolya *et al.*, 1997); *Thermobifida fusca* ATCC 27730 was the reference strain. They were preserved in lyophilized ampoules as mixtures of freeze-dried spores and vegetative mycelium. Working stock cultures were maintained on MN300 cellulose agar (Kukolya *et al.*, 1997). The inocula used for physiological tests were grown in Luria-Bertani medium or saccharose broth (0.5 g yeast extract, 1 g NaNO₃, 1 g K₂HPO₄, 0.3 g KCl, 0.05 g MgSO₄·7H₂O, 10 g saccharose, 1 l distilled water, pH 7.4). The biomass used for extraction of DNA was produced in Luria-Bertani broth as shaken culture (200 r.p.m. at 50 °C).

Morphological observations. Colony morphology was examined on cultures grown for 7 days at 40 °C (strain K21) or 50 °C (strains TB100^T, TB108, TB110) on MN300 agar. Micro-morphology and sporulation were determined by light microscopy and scanning electron microscopy. Mycelia scraped off the agar plates after 4 days incubation were fixed overnight in Plumel's cacodylate buffer (pH 7.0, containing 2.5% glutaraldehyde), dehydrated through a graded acetone series, critical-point dried from liquid CO₂, and sputter coated with gold. The samples were examined with a Zeiss EM910 electron microscope at 40 kV.

Physiological tests. Physiological tests, including production of acid from carbohydrates, pH sensitivity and spore heat resistance, were determined as described by McCarthy (1989). Growth temperature ranges were determined by

culturing the strains on MN300 agar at 28, 30, 35, 40, 44, 50, 55, 57, 61, 66 and 69 °C for 7 days. Antibiotic sensitivity was tested on saccharose broth at concentrations of 1.25, 2.5, 5.0, 10.0 and 20.0 µg ml⁻¹.

Carbon-source utilization. Utilization of carbon sources was tested by using the mineral salts medium of McCarthy (1989). Each carbon source, sterilized by membrane filtration, was added to the medium to give a final concentration of 1% (w/v). After 7 days incubation at 40 °C (for strain K21) or 50 °C (for the other strains), growth was compared with that measured on the negative and positive control plates (basal medium without a carbon source or supplemented with glucose, respectively).

Degradative tests. Polysaccharide-degradation activities were determined on mineral salts plates containing 1% (w/v) crystalline cellulose (MN300; Machery Nagel), 0.2% colloidal chitin, 0.2% medium viscosity CM-cellulose (Sigma), 0.2% birch-wood xylan (Sigma) or 1% maize starch (Sigma). After 48 h incubation at the optimum temperature, CM-cellulose and xylan hydrolyses were visualized by staining with Congo red (Sazci *et al.*, 1986); starch degradation was visualized by staining the plates with 5 × diluted Gram's iodine for 5 min. Crystalline cellulose- and chitin-decomposition activities were detected as clearing zones around the colonies after 7 days incubation. Lignin-solubilization activity was measured by the method of Trigo & Ball (1994) by using 1% (w/v) ball-milled straw as the carbon source. The formation of an acid-precipitable lignocarbhydrate complex was measured spectrophotometrically according to Trigo & Ball (1994). Degradation of elastin, Tween 20, Tween 80, casein and arbutin was determined by using the procedure of McCarthy & Cross (1984).

Chemotaxonomic characterization. The cell-wall diamino acid and the sugar patterns of the four strains were determined from whole-cell hydrolysates as described by Hasegawa & Takizawa (1983). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977) and the profile was analysed by HPLC (HP 1100) (Groth *et al.*, 1997). Cellular fatty acids were extracted according to Stead *et al.* (1992) and were analysed by GC (Groth *et al.*, 1996). Polar lipids were determined according to the method described by Minnikin *et al.* (1979). Mycolic acids were tested as recommended by Minnikin *et al.* (1975).

DNA base composition. The G + C content (mol%) of the DNA was determined spectrophotometrically from the midpoint of thermal denaturation in 0.1 × standard saline citrate by the method of English *et al.* (1999), using *Cellulomonas flavigena* DSM 20109 as the reference strain.

DNA-DNA hybridization. The reaction mixture consisted of 75 µg sheared (400–600 bp) DNA ml⁻¹ in 3 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na₃-citrate at pH 7.6) and 25% DMSO. DNA was denatured for 10 min at 95 °C. Reassociations were run at 60 °C (*T*_m 23 °C) for 300 min. The standard error was 4%. The absorbance of the DNA was recorded at 258 nm. Absorbance signals were logged at 1 min intervals. The *C*₀*t*_{0.5} values and the percentage DNA relatedness were calculated as described by Labeda (1992).

16S rDNA sequencing. DNA was extracted from cells according to the methods of Hunter (1985). PCR amplification of 16S rDNA was performed as described by Rainey *et al.* (1996). PCR products were purified with the Prep-A-Gene kit (Bio-Rad) according to the manufacturer's

instructions. Sequencing reactions were performed using the BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems) according to the manufacturer's instructions. Partial sequences of the first 500 bp of the 16S rDNA were initiated with the 531r conservative eubacterial primer. The almost complete sequence of the 16S rDNA of strain TB100^T was determined by using the primers 357f, 531r, 803f and 1114f. Sequences obtained by an ABI PRISM 310 automated sequencer were aligned manually and analysed with the ARB phylogenetic package. Reference sequences were taken from the Ribosomal Database Project (Maidak *et al.*, 1999). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), the De Soete tree fit (De Soete, 1983), and the maximum-parsimony (Fitch, 1971) methods. Bootstrapping was used to determine the confidence level of the tree topology.

RESULTS AND DISCUSSION

Cultural, physiological and biochemical characteristics

All strains shared a number of phenotypic properties consistent with their assignment to the genus *Thermobifida* (Table 1). They formed highly branched substrate mycelium and heat-sensitive spores ($1\text{--}1.3 \times 0.6 \mu\text{m}$) on aerial hyphae, on the tips of branched sporophores (Fig. 1). Repeated sporophore branching resulted in the formation of dense spore clusters (Fig. 2), as revealed by scanning electron microscopy. The surfaces of the spores became scaly upon maturation, giving a pine-cone appearance for these structures. Growth temperature ranges were 28–50 °C for strain K21, 44–57 °C for strain TB100^T, 44–61 °C for strain

Fig. 1. Spores of strain TB100^T of *Thermobifida cellulolytica* sp. nov. formed on aerial hyphae, on the tips of branched sporophores. Bar, 1 μm .

TB108 and 44–66 °C for TB110 and the reference strain, *Thermobifida fusca* ATCC 27730. All strains tolerated a pH increase up to 10.0, a value approaching that actually measured in the sampling region of the compost pile where these actinomycetes were found. Of the carbon sources tested, all four strains were able

Table 1. Phenotypic characteristics of *Thermobifida cellulolytica* strains isolated from manure compost and of the reference strain *Thermobifida fusca* (ATCC 27730^T)

Characteristic	K21	TB100 ^T	TB108	TB110	<i>T. fusca</i> ATCC 27730 ^T
Abundant white aerial mycelium	—	+	+	+	+
Soluble pigments on MN300 agar	Pale brown	—	—	Yellowish-brown	—
Spore size (μm)	1×0.5	1.1×0.6	1.3×0.6	1.1×0.6	1×0.6
Degradation of agar-agar	—	—	—	+	+
Growth on sole carbon sources:					
Adonitol	+	+	—	—	+
L-Arabinose	—	—	+	—	—
Glycerol*	—	—	—	—	+
L-Rhamnose	—	+	+	—	—
D-Melibiose	+	—	+	—	—
Raffinose	—	+	+	+	+
D-Ribose	—	+	+	+	—
Acid produced from glucose*	—	—	—	—	+
Growth with:					
Erythromycin (20 $\mu\text{g ml}^{-1}$)*	+	+	+	+	—
Tetracycline HCl (2.5 $\mu\text{g ml}^{-1}$)	—	+	—	—	—

* Traits that are consequently different in the newly described strains and in the type strain of the genus *Thermobifida* (*Thermobifida fusca* ATCC 27730^T).

Fig. 2. Repeated sporophore branching resulting in the formation of dense spore clusters in strain TB110 of *Thermobifida cellulolytica* sp. nov. Bar, 1 µm. Stereoscopic images of spore clusters are available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>).

to utilize D-xylose, D-mannose, D-glucose, D-galactose, D-fructose, cellobiose, maltose, lactose, sucrose, trehalose and melezitose, but not L-sorbose, glycerol, *meso*-inositol, dulcitol or D-sorbitol. The strains degraded elastin, Tween 20, Tween 80, arbutin, casein,

CM-cellulose, xylan and starch, and were able to solubilize lignocellulose substrate. All strains showed high cellulase activity on crystalline cellulose, and the clearing zones observed on MN300 agar plates indicated the presence of a complete cellulase enzyme system. Chitinase activity was absent. *Thermobifida fusca* and *Thermobifida alba* are also efficient cellulolytic organisms and lack chitinase activity (McCarthy, 1989). The strains were all susceptible to novobiocin (10 µg ml⁻¹), ampicillin (10 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹), kanamycin (2.5 µg ml⁻¹), streptomycin (2.5 µg ml⁻¹), tetracycline hydrochloride (5 µg ml⁻¹), apramycin (2.5 µg ml⁻¹), thiostrepton (1.25 µg ml⁻¹), penicillin (10 µg ml⁻¹) and amoxycillin (10 µg ml⁻¹). They were resistant to erythromycin at 20 µg ml⁻¹.

Chemotaxonomic properties

The chemotaxonomic properties are summarized in Table 2. The cell wall contained *meso*-diaminopimelic acid as the murein diamino acid, and no characteristic sugar was detected, indicating the presence of wall chemotype III and sugar pattern type C. The major isoprenoid quinones were octa- and hexahydrogenated menaquinones with 10 isoprenoid units [MK-10(H₈), MK-10(H₆)]. According to Kroppenstedt (1985), these values indicate menaquinone pattern type 4D, which is a diagnostic trait for the genus *Thermobifida*. Substantial amounts of uncommon menaquinones [MK-11(H₆), MK-8(H₆)] were also present in all strains, whereas the MK-10(H₄) fraction was identified only in the three thermophilic strains. This menaquinone profile differs characteristically from that of the two known species of *Thermobifida*, *Thermobifida alba* and

Table 2. Chemotaxonomic traits of *Thermobifida* strains isolated from manure compost

Polar lipid analysis: all strains had phosphatidylethanolamine and glycolipid.

Strain	K21	TB100 [†]	TB108	TB110
Cellular fatty acid analysis*				
15:0 iso	4.3	18.5	16.61	2.46
15:0 anteiso	1.8	3.07	3.35	1.08
16:0 iso	23.92	14.81	13.98	20.12
16:0	2.3	0.5	0.2	4.35
17:0 iso	32.14	—	—	—
17:0 anteiso	—	32.2	33.74	35.84
17:0 cyclo9–10	21.64	19.6	24.62	15.91
17:0	1.1	2.5	0.26	2.35
18:2 <i>cis</i> 9, 12	7.03	4.95	1.65	7.17
18:0	4.59	—	—	9.78
Total (%)	98.82	96.13	94.41	99.06
Analysis of isoprenoid quinones [†]	MK-8(H ₆), MK-11(H ₆)	MK-10(H ₄), MK-8(H ₆), MK-11(H ₆), MK-8(H ₄)	MK-10(H ₄), MK-11(H ₆), MK-8(H ₆)	MK-11(H ₆), MK-8(H ₆), MK-10(H ₄)

* Values less than 1% are not shown.

[†] All strains had MK-10(H₈) and MK-10(H₆) as major components.

Fig. 3. Neighbour-joining tree based on 16S rDNA sequences of *Thermobifida cellulolytica* sp. nov., other *Thermobifida* strains, and their close relatives. Bootstrap values are calculated from 1000 repeats. Bar, 10 nucleotide substitutions per 100 nucleotides.

Thermobifida fusca. In *Thermobifida fusca*, besides the dominant menaquinones, MK-10(H₆) and MK-10(H₈), substantial amounts of MK-11(H₆) and MK-11(H₈) were recorded, whereas *Thermobifida alba* contained MK-10(H₁₀) as the differentiating compound (Kroppenstedt & Goodfellow, 1992). The peak area ratios of the dominant menaquinones showed no significant change when different incubation temperatures (45 and 55 °C) were used, whereas the ratios of minor menaquinone components were influenced by temperature. All strains had PLII-type phospholipid composition according to Lechevalier *et al.* (1981): phosphatidylethanolamine, glycolipid and some unidentified phospholipids were identified. The diagnostic fatty acid pattern was similar in all strains. The amount of the C₁₅-iso + C₁₇-anteiso fraction was over 30%, and the hydroxy and 10-methyl fractions were undetectable, indicating the presence of fatty acid pattern type 3e, which is typical of actinomycetes belonging to the genus *Thermobifida* (Kroppenstedt & Goodfellow, 1992). No mycolic acids were detected.

Sequence comparison and phylogenetic analysis of 16S rRNA gene

Strains studied in this work exhibited 99.8–100% sequence similarity based on the analysis of the first 500 bp of the 16S rRNA gene, indicating a close interrelationship. A nearly complete sequence of the 16S rRNA gene from strain TB100^T was determined. This sequence exhibited 97.4 and 97.8% similarity to the 16S rRNA gene of *Thermobifida alba* and *Thermobifida fusca*, respectively. The 16S rRNA sequences of *Thermobifida alba* and *Thermobifida fusca* share 97.5% similarity. These values support the separation of strain TB100^T from the two previously described

Thermobifida species. The topologies of the phylogenetic trees were very similar, and strain TB100^T clustered together with the genus *Thermobifida* with a bootstrap value of 87%. The exact branching order of the two *Thermobifida* species and strain TB100^T, however, was difficult to identify. In the tree prepared by maximum-parsimony analysis, *Thermobifida alba* was more closely related to strain TB100^T than to *Thermobifida fusca*, but, in the neighbour-joining and the De Soete tree, *Thermobifida alba* and *Thermobifida fusca* branched together and strain TB100^T formed a distinct cluster (Fig. 3). The bootstrap values of this branching point were similarly low when either the neighbour-joining or the maximum-parsimony treeing method was used.

DNA base composition

Thus far, data on the G + C contents of the DNAs of any members of the genus *Thermobifida* have been lacking. In the present experiments, the G + C contents of strains K21, TB100^T, TB110 and TB108 were determined as 70.6, 70.2, 69.8 and 70.3 mol%, respectively. The G + C content of the reference strain, *Thermobifida fusca* ATCC 27730^T, was significantly lower, amounting to 66.5 mol%, a value comparable to the mean G + C content of the genes that had until now been cloned and sequenced from *Thermobifida fusca* (Jung *et al.*, 1993; Lao *et al.*, 1991).

DNA–DNA relatedness

DNA–DNA reassociation studies were used to confirm the species status of the four new isolates in relation to the other two *Thermobifida* species described thus far. The results of DNA–DNA

Table 3. Percentage DNA–DNA relatedness among the type strains of *Thermobifida fusca* ATCC 27730^T and *Thermobifida alba* DSM 43795^T, as well as the newly described four strains of *Thermobifida*

Strains: 1, *T. fusca* (= ATCC 27730^T); 2, *T. alba* (= DSM 43795^T); 3, TB110; 4, TB100^T (= DSM 44535^T); 5, TB108; 6, K21. Method used: thermal renaturation.

Strain	1	2	3	4	5	6
1	100					
2	33.2	100				
3	48.4	56.8	100			
4	48.1	57	99.1	100		
5	49.3	57.2	98.4	98.5	100	
6	46.3	53.7	94.6	95.3	96.2	100

reassociation studies are presented in Table 3. These results confirmed the high degree of relatedness among the four new isolates found by 16S rDNA sequence comparison. The DNA–DNA homology values were greater than 94.6%, indicating that all four strains belong to a single species.

The new species status of these four isolates was further supported by the low reassociation values obtained in comparisons with *Thermobifida fusca* ATCC 27730^T (46.3–49.3%) and *Thermobifida alba* DSM 43795^T (53.7–57.2%). According to Wayne *et al.* (1987), two strains with less than 70% DNA homology belong to different species.

The morphological, physiological and chemotaxonomic properties, as well as the 16S rDNA sequence similarity, strongly support the affiliation of strains K21, TB100^T, TB108 and TB110 to the genus *Thermobifida* (Zhang *et al.*, 1998). We assign these four strains to a single species because of the high levels of similarity found in their 16S rDNA sequences, menaquinone profiles and DNA G+C contents, as well as the strong relative homology observed among them in DNA–DNA hybridization experiments. Differences in colony morphology and physiological traits among the four strains remained within the boundaries of a species. On the other hand, these strains differ characteristically from *Thermobifida fusca* and *Thermobifida alba* in the above-mentioned traits. On the basis of these results, these four isolates were assigned to a new species named *Thermobifida cellulolytica*.

Description of *Thermobifida cellulolytica* sp. nov.

Thermobifida cellulolytica (cel.lu.lo.ly'ti.ca. N.L. n. *cellulosum* cellulose; Gr. adj. *lyticus* dissolving; N.L. fem. adj. *cellulolytica* cellulose-dissolving).

Aerobic, Gram-positive actinobacterium. Colonies on agar media bear abundant white aerial mycelium. Sporophores branch repeatedly to form dense spore clusters on aerial mycelium. The surfaces of the oval

(1–1.3 × 0.6 µm), heat-sensitive (90 °C, 10 min) spores are scale-like. The growth temperature ranges of the thermotolerant and thermophilic strains are 28–45 and 40–65 °C, respectively. Crystalline cellulose, gelatin, starch, arbutin, elastin, xylan, Tween 20 and Tween 80, but not chitin, are degraded. There is no acid production from glucose. D-Xylose, D-mannose, D-glucose, D-galactose, D-fructose, cellobiose, maltose, lactose, sucrose, trehalose and melezitose are used as sole carbon sources, but L-sorbose, glycerol, *meso*-inositol, dulcitol and D-sorbitol are not. Cultures grown on lignocellulose substrate produce endoglucanase, cellobiohydrolase, endoxylanase enzymes and are capable of solubilizing lignocarbohydrates. Growth is inhibited by novobiocin (10 µg ml⁻¹), ampicillin (10 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹), kanamycin (2.5 µg ml⁻¹), streptomycin (2.5 µg ml⁻¹), tetracycline hydrochloride (5 µg ml⁻¹), apramycin (2.5 µg ml⁻¹), thiostrepton (1.25 µg ml⁻¹), penicillin (10 µg ml⁻¹) and amoxycillin (10 µg ml⁻¹). Resistant to erythromycin at 20 µg ml⁻¹. The cell wall type is chemotype III, containing *meso*-diminopimelic acid without characteristic sugars. The menaquinone pattern is type 4D; the dominant menaquinones are MK-10(H₈) and MK-10(H₆) with some uncommon menaquinones, including MK-11(H₆), MK-8(H₆) and MK-8(H₄). The phospholipid pattern is type II (phosphatidylethanolamine, glycolipid) and the fatty acid pattern is type 3e (16:0 iso, 17:0 iso, 17:0 anteiso, 17:0 cyclo 9–10 type fatty acids are predominant). The G+C content of the DNA is between 70 and 71 mol%. Isolated from overheated compost by J. Kukolya in 1997 at Gödöllő, Hungary. The type strain is strain TB100^T (= DSM 44535^T = NCAIM B01997^T).

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