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***Thermoactinomyces khenchelensis* sp. nov., a filamentous bacterium isolated from soil sediment of a terrestrial hot spring**

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Abdelhadi Lahoum · Abdelghani Zitouni · Carol Verheecke ·
Florence Mathieu · Peter Schumann · Cathrin Spröer ·
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Abstract A novel thermophilic filamentous bacterium, designated strain T36^T, was isolated from soil sediment sample from a hot spring source collected in Khenchela province, Algeria. Strain T36^T was identified as a member of the genus *Thermoactinomyces* by a polyphasic approach. Strain T36^T was observed to form white aerial mycelium and non-coloured to pale yellow substrate mycelium, both producing endospores, sessile or borne by short sporophores. The optimum growth temperature and pH were found to be 37–55 °C and 7.0–9.0, respectively and the optimum NaCl concentration for growth was found to be 0–7 % (w/v). The diagnostic diamino acid in the

cell wall peptidoglycan was identified as *meso*-diaminopimelic acid. The predominant menaquinone of strain T36^T was identified as MK-7 (H₀). The major fatty acids were found to be iso-C_{15:0} and iso-C_{17:0}. The phospholipids detected were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphoglycolipid. The chemotaxonomic properties of strain T36^T are consistent with those shared by members of the genus *Thermoactinomyces*. 16S rRNA gene sequence analysis indicated that the sequence similarities between strain T36^T and *Thermoactinomyces* species with validly published names were less than 98 %. Based on the combined genotypic and phenotypic evidence, it is proposed that strain T36^T should be classified as representative of a novel species, for which the name

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Thermoactinomyces khenchelensis sp. nov. is proposed. The type strain is T36^T (=DSM 45951^T = CECT 8579^T).

Keywords *Thermoactinomyces* ·
Thermoactinomyces khenchelensis sp. nov. ·
Thermophilic filamentous bacterium · Hot spring ·
Polyphasic taxonomy

Introduction

The genus *Thermoactinomyces* within the family *Thermoactinomycetaceae* was described for the first time by Tsilinsky (1899) with the single species *Thermoactinomyces vulgaris*. Several species of *Thermoactinomyces* have been described since but most of them have been reclassified in other genera (Yoon et al. 2005). At time of writing, the genus *Thermoactinomyces* comprises only four species with validly published names: *Thermoactinomyces vulgaris* (Tsilinsky 1899), *Thermoactinomyces intermedius* (Kurup et al. 1980), *Thermoactinomyces daqus* (Yao et al. 2014) and *Thermoactinomyces guangxiensis* (Wu et al. 2015). Members of the genus *Thermoactinomyces* are Gram-positive, aerobic, thermophilic, filamentous bacteria. Endospores are sessile and are formed singly on both aerial and substrate hyphae or on unbranched short sporophores. Growth occurs mainly at 55 °C but not at 30 °C (Lacey and Cross 1989). The predominant menaquinone is MK-7. The major fatty acid is iso-C_{15:0}, and significant amounts of iso-C_{17:0} are also present (Yoon et al. 2005).

In this study, a novel thermophilic filamentous bacterium, designated strain T36^T, was isolated from a soil sediment sample and, based on polyphasic taxonomic analysis, classified as representative of a novel species, for which the name *Thermoactinomyces khenchelensis* sp. nov. is proposed.

Materials and methods

Isolation and maintenance of strains

Strain T36^T was isolated from a soil sediment sample collected from a hot spring (35°25'N, 7°8'E) located in the Aurès Mountains (part of the Atlas Mountains), Khenchela province in North-East Algeria.

Isolation was carried out using a dilution-agar plating method on nutrient agar medium (Waksman 1961) supplemented with cycloheximide (50 µg ml⁻¹) to reduce fungal growth. The pH was adjusted to 7.0 by using autoclaved NaOH. After incubation at 52 °C for 1 week, the *Thermoactinomyces*-like strain was picked and purified, and also stored at 4 °C on the same medium.

Strain T36^T has been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany, as strain DSM 45951^T and in Spanish Type Culture Collection (CECT), Spain, as strain CECT 8579^T.

The type strains *T. daqus* DSM 45914^T, *T. vulgaris* DSM 43016^T and *T. intermedius* DSM 43846^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany, and used as reference strains in comparative testing.

Phenotypic characterisation

Cultural characteristics were investigated after 4 days of incubation at 52 °C on ISP 2 and ISP 4 media of the International *Streptomyces* Project (Shirling and Gottlieb 1966) and also on nutrient agar medium (Waksman 1961). The degree of growth was determined and the colours of the substrate and aerial mycelia and any soluble pigments produced were determined by comparison with ISCC-NBS colour charts (Kelly and Judd 1976). The morphological characteristics of strain T36^T were observed by light microscopy (Model B1; Motic) and scanning electron microscopy (JSM-7100F; JEOL) after incubation on nutrient agar at 50 °C for 7 days.

Strain T36^T was characterised by using a range of physiological tests. All tests were performed at pH 8.0 (except those of pH test) which is the optimum pH of growth. Degradation of adenine, gelatin, guanine, hypoxanthine, starch, testosterone, Tween 80, tyrosine and xanthine, and also coagulation and peptonization of milk were studied as described by Goodfellow (1971) and Marchal et al. (1987). Production of nitrate reductase was determined according to the method of Marchal et al. (1987). Production of melanoid pigments was tested on ISP 6 and ISP 7 media (Shirling and Gottlieb 1966). Growth in the presence of novobiocin (25 µg ml⁻¹), at different temperatures (30, 37, 45, 50, 55 and 60 °C), at various values of pH (5, 6, 7, 8, 9 and 10) and also in the presence of

different concentrations of NaCl (0, 1, 2, 3, 4, 5, 6, 7 and 8 %; w/v) were determined by using nutrient agar medium, after incubating for 7 days at 52 °C.

Chemical analysis of cell constituents

For chemical analysis, the biomass of strain T36^T was obtained by cultivating the cells in shake flasks at 52 °C for 2 days on a rotary shaker (250 rpm) using tryptic soy broth (TSB, Oxoid) (pH 8). Biomass was harvested by centrifugation at 3500 rpm and washed twice with distilled water. The isomeric form of diaminopimelic acid in the cell wall was ascertained as described by Becker et al. (1964). Phospholipids were analysed according to the method of Minnikin et al. (1977). The fatty acid profile was determined by the method of Sasser (1990), using the Microbial Identification System (MIDI) Sherlock software version 6.1 (method TSBA40, TSBA6 database). Menaquinones were isolated according to the method of Minnikin and O'Donnell (1984) and were analysed by HPLC (Kroppenstedt 1982, 1985).

Phylogenetic analyses

Strain T36^T was grown in tryptic soy broth (TSB, Oxoid; pH 8) and genomic DNA was extracted with a DNA extraction kit (MasterPure Gram Positive DNA Purification kit; Epicentre Biotechnologies). PCR amplification of the 16S rRNA gene was performed as described by Rainey et al. (1996). PCR products were purified with a PCR product purification kit (Qiagen). The primers used for sequencing were as listed in Coenye et al. (1999). The sequence obtained was compared with sequences present in the public sequence databases as well as with the EzTaxon-e server (Kim et al. 2012). Phylogenetic analyses were conducted using MEGA version 5 (Tamura et al. 2011). The 16S rRNA gene sequence of strain T36^T was aligned against neighbouring nucleotide sequences using CLUSTAL W (with default parameters) (Larkin et al. 2007). Phylogenetic trees were reconstructed by using the neighbour-joining method (Saitou and Nei 1987) with the model of Jukes and Cantor (1969), maximum-likelihood (Felsenstein 1981) with Kimura 2-parameter (Kimura 1980) model and maximum-parsimony (Fitch 1977) methods. The topology of the trees was evaluated by bootstrap analysis based on 1000 replicates (Felsenstein 1985).

Results and discussion

Strain T36^T was found to show good growth on ISP 2, nutrient agar and Bennett's agar media, but no growth was observed on ISP 4 medium after 7 days of incubation at 52 °C. White aerial mycelium was found to be produced on ISP 2 and Bennett's agar media, but not well developed on nutrient agar medium. The substrate mycelium was observed to be white to pale brownish yellow on ISP 2 medium, non-coloured to pale yellow on nutrient agar medium and non-coloured on Bennett's agar medium. Sporulation was found to be weak on ISP 2, good on nutrient agar medium and very good on Bennett's agar medium. Endospores (0.8–1.0 µm in diameter) round in shape, were observed to be sessile and to form singly on both aerial and substrate mycelia, and sometimes are produced singly on very short sporophores (Fig. 1). No diffusible pigment was detected on any tested media.

Strain T36^T was determined to contain *meso*-diaminopimelic acid in its cell wall and was found to possess diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphoglycolipid, unknown phospholipid and two unknown polar lipids (Fig. S1). The predominant menaquinone was determined to be MK-7 (H₀) (87.5 %); MK-8 (H₀) (9.7 %) and unknown menaquinones were also detected. The fatty acids profile was found to be composed as follows: iso-C_{15:0} (66.5 %), iso-C_{17:0} (17.5 %), anteiso-C_{15:0} (6.2 %), iso-C_{16:0} (4.2 %) and iso-C_{13:0} (1.4 %). The morphological and

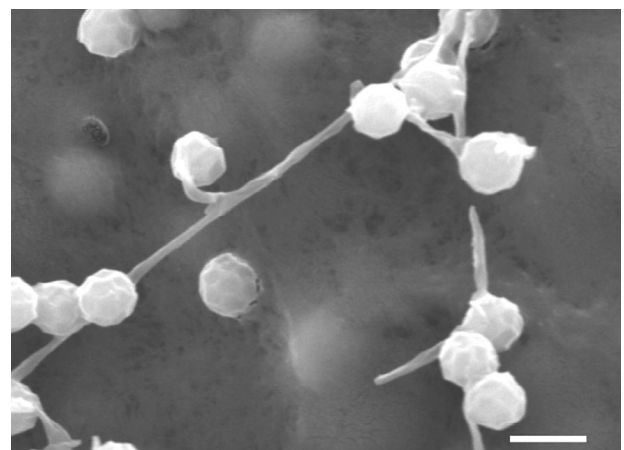


Fig. 1 Scanning electron microscopy of strain T36^T grown on nutrient agar for 7 days at 50 °C, showing round spores carried by short sporophores. Bar 1 µm

Table 1 Differential phenotypic and chemotaxonomic characteristics of strain T36^T and type strains of recognized species of the genus *Thermoactinomyces*

Characteristics	1	2	3	4	5
Aerial mycelium	White	White	Yellow–white	White	White
Sporophores	Short	Long	Short	Short	Short
pH range	7.0–9.0	6.0–11.0	5.0–9.0	5.0–8.0	5.0–8.0
Temperature range (°C)	37–55	37–55	45–60	37–60	37–65
Nitrate reduction	+	–	–	+	–
Gelatin liquefaction	+	+	+	+	–
Milk peptonization	–	+	+	+	+
Degradation of					
Hypoxanthine	+	+	–	–	–
Utilization of					
D-Fructose	+	+	+	–	–
D-Glucose	+	+	–	–	–
D-Lactose	–	–	–	+	–
D-Mannitol	–	–	+	+	–
Maltose	+	+	+	–	–
D-Trehalose	–	–	+	–	–
D-Raffinose	–	+	+	+	–
L-Rhamnose	+	+	+	–	–
L-Threonine	–	+	–	–	–
L-Serine	–	+	–	–	–
Growth in the presence of 5 % (w/v) NaCl	+	–	–	–	–
Phospholipids	DPG, PE, PG, PI, PIM	DPG, PE, PG, OH-PE, NPG, PL, GLS	DPG, PE, PG, PI, PIM, L, PL	DPG, PE, PG, PL	DPG, PE, PG, PI, PIM, PL

Strains 1 strain T36^T, 2 *T. guangxiensis* ATCC BAA-2630^T, 3 *T. daqus* DSM 45914^T, 4 *T. vulgaris* DSM 43016^T, 5 *T. intermedius* DSM 43846^T

Data for physiological tests were from this study, except those of *T. guangxiensis* ATCC BAA-2630^T (taken from Wu et al. 2015). Data for phospholipid analysis were taken from Wu et al. (2015), except for strain T36^T (this study)

+ positive, – negative

DPG Diphosphatidylglycerol, PE phosphatidylethanolamine, OH-PE hydroxyphosphatidylethanolamine, PG phosphatidylglycerol, PI phosphatidylinositol, PIM phosphatidylinositol mannoside, NPG ninhydrin-positive glycopospholipid, L unknown lipids, PL unknown phospholipids, GLS glycolipids

chemical characteristics described above clearly support the placement of strain T36^T within the genus *Thermoactinomyces*.

Temperature and pH ranges for growth were found to be 37–55 °C and pH 7.0–9.0, with optima at 50–55 °C and pH 8.0, respectively. The NaCl concentration range for growth is 0–7 %, with optimal growth occurring at 0–2 %. Strain T36^T was found to be resistant to novobiocin (25 µg ml⁻¹). Detailed results of the physiological and biochemical analyses are given in Table 1 (in comparison with all *Thermoactinomyces* species) and in the species description

below. It is evident from Table 1 that there are several phenotypic characteristics that clearly separate strain T36^T from the related recognised species in the genus *Thermoactinomyces*.

Phylogenetic analysis of an almost complete 16S rRNA gene sequence (1508 bp, GenBank accession number KT277569) showed that strain T36^T is related to members of the genus *Thermoactinomyces* and exhibits high 16S rRNA gene sequence similarity to *T. vulgaris* (97.9 %), *T. intermedius* (97.7 %), *T. daqus* (95.2 %) and *T. guangxiensis* (94.6 %). Meier-Kolthoff et al. (2013) showed that

the DNA–DNA hybridization should be mandatory only above a similarity percentage of 98.2 % (based on 16S rRNA gene sequences). More recently, Kim et al. (2014) reported that 98.65 % 16S rRNA gene sequence similarity can be used as the threshold for differentiating two bacterial species. Consequently, DNA–DNA hybridization is not considered necessary to distinguish strain T36^T from *T. vulgaris* (97.9 %) or the other type strains. The phylogenetic relationship between strain T36^T and the other *Thermoactinomyces* species is seen in the neighbour-joining (Fig. 2), maximum parsimony (Fig. S2) and maximum-likelihood (Fig. S3) dendrograms.

On the basis of polyphasic taxonomic evidence, it is concluded that strain T36^T represents a novel species of the genus *Thermoactinomyces*, for which the name *Thermoactinomyces khenchelensis* sp. nov. is proposed.

Description of *Thermoactinomyces khenchelensis* sp. nov

Thermoactinomyces khenchelensis (khen.chel.en' sis. N.L. masc. adj. *khenchelensis* referring to Khenchela, the source of the soil from which the type strain T36^T was isolated).

Thermophilic filamentous bacterium that produces round sessile endospores form singly on both aerial

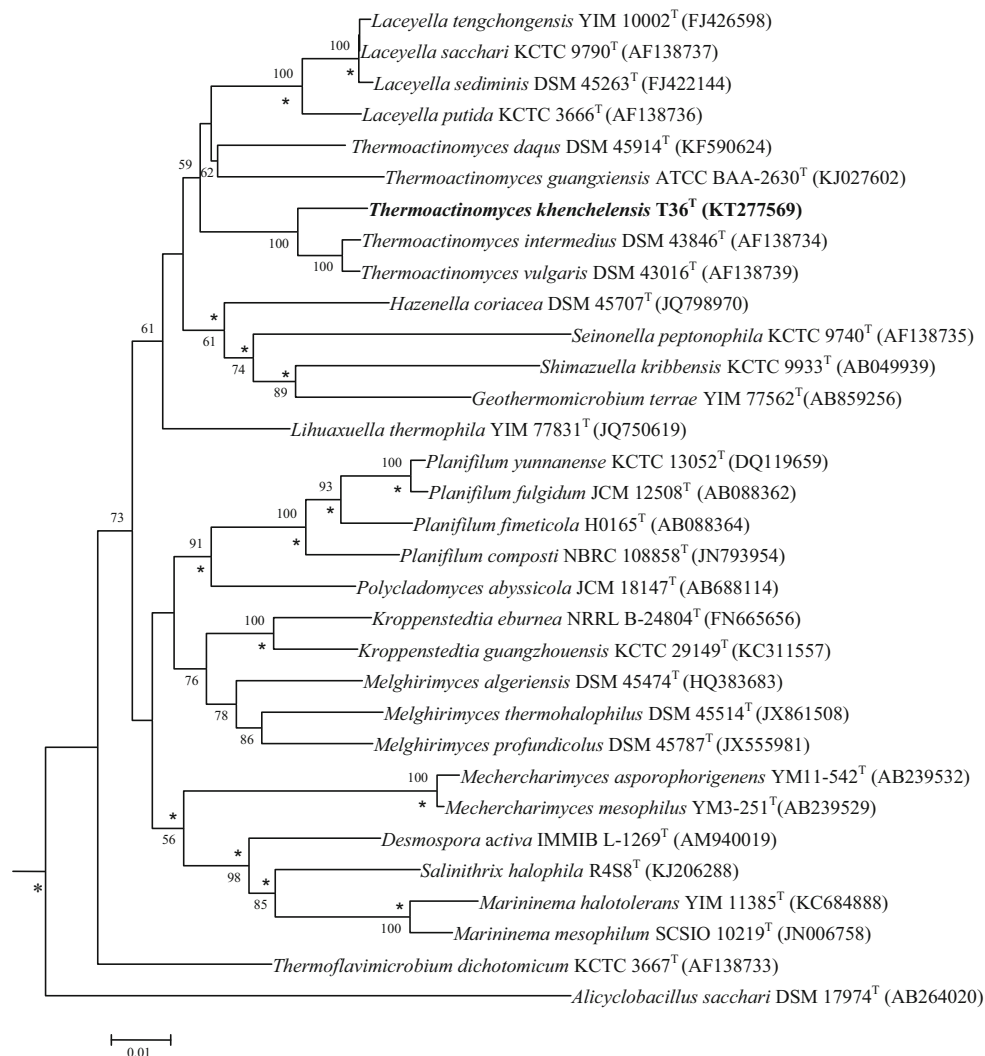


Fig. 2 Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain T36^T (1508 bp) and the species of the related genera of the family *Thermoactinomycetaceae*. Asterisks indicate branches that are conserved when the neighbour-joining, maximum-parsimony

and maximum-likelihood methods were used in constructing phylogenetic trees. Numbers at the nodes are bootstrap values (>50 %), expressed as a percentage of 1000 resamplings. *Alicyclobacillus sacchari* DSM 17974^T was used as the outgroup. Bar 0.01 nucleotide substitutions per site

and substrate mycelia; endospores are sometimes produced singly on very short sporophores. Aerial mycelium is abundant and shows a white colour on ISP2 and Bennett's agar media. White to pale yellow substrate mycelium is observed on ISP 2, nutrient agar and Bennett's media. Diffusible pigments are not produced on any media tested here. Temperature and pH ranges for growth are 37–55 °C and pH 7.0–9.0, with optima at 50–55 °C and pH 8.0. The NaCl concentration range for growth is 0–7 %, with optimal growth occurring at 0–2 %. Utilises D-glucose, D-fructose, maltose, L-rhamnose, D-ribose and sucrose but not L-arabinose, D-cellobiose, D-galactose, myo-inositol, D-lactose, D-mannitol, D-melibiose, methyl α -D-glucoside, D-raffinose, D-sorbitol, D-trehalose or D-xylose. Positive for adenine, gelatin, hypoxanthine and Tween 80 hydrolysis, and also milk coagulation, but negative for arbutin, esculin, guanine, tyrosine, xanthine and starch hydrolysis, and milk peptonization. L-alanine, L-serine, L-proline and L-threonine are not used as a source of nitrogen. Nitrate reductase is produced.

The diagnostic phospholipid detected is phosphatidylethanolamine, along with diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphoglycolipid. The predominant menaquinone is MK-7 (H₀). Major fatty acids are iso-C_{15:0} and iso-C_{17:0}.

The type strain is T36^T (=DSM 45951^T = CECT 8579^T), which was isolated from soil sediment of a hot spring source located in Khenchela province (Algeria). The GenBank accession number for the 16S rRNA gene sequence of strain T36^T is KT277569.

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