Note

Streptomyces mexicanus sp. nov., a xylanolytic micro-organism isolated from soil

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The taxonomic position of a thermophilic actinomycete strain isolated from soil was examined using a polyphasic approach. The strain, designated CH-M-1035^T, was assigned to the genus *Streptomyces* on the basis of chemical and morphological criteria. It formed *Rectiflexibiles* aerial hyphae that carried long chains of rounded, smooth spores. The almost complete nucleotide sequence of the 16S rRNA gene of strain CH-M-1035^T was determined and its comparison with the 16S rDNA sequences of previously studied streptomycetes confirmed the assignment of the novel strain to the genus *Streptomyces*. Strain CH-M-1035^T clustered with species belonging to the *Streptomyces thermodiastaticus* clade in the 16S-rDNA-based phylogenetic tree. However, the phenotypic properties of strain CH-M-1035^T differed from those of the recognized species within this clade. Therefore, it is proposed that strain CH-M-1035^T be classified as a novel species within the genus *Streptomyces*, as *Streptomyces mexicanus* (type strain CH-M-1035^T = DSM 41796^T = BM-B-384^T = NRRL B-24196^T).

The streptomycetes are widely used in industry, due to their ability to produce numerous chemical compounds, including antibiotics, enzymes and anti-tumour agents (Bérdy, 1995). Currently, more attention is paid to the thermophilic streptomycetes, due to their potential importance in microbial technology. These micro-organisms grow well at 50 °C (Kim *et al.*, 1999), and can be distinguished from mesophilic streptomycetes on the basis of numerical phenotypic analyses (Goodfellow *et al.*, 1987; O'Donnell *et al.*, 1993). However, the thermophilic streptomycetes fall into distinct evolutionary clades, upon analyses based on 16S rDNA sequence data (Kim, D. *et al.*, 1996; Kim, S. B. *et al.*, 1998; Kim, B. *et al.*, 1999).

During a routine screening of strains isolated from a sugar-cane field in the State of Morelos, Mexico, an actinomycete producing thermostable xylanolytic enzymes was isolated. This strain, designated CH-M-1035^T, was shown to present a colonial morphology consistent with its assignment to the genus *Streptomyces* (Flores *et al.*, 1996, 1997). In the

present investigation, strain CH-M-1035^T was the subject of a polyphasic taxonomic study, to establish whether it represented a novel species of the genus *Streptomyces*.

Strain CH-M-1035^T was isolated on minimal medium containing 2 g KH₂PO₄ l⁻¹, 1.4 g (NH₄)₂SO₄ l⁻¹ and 5 g oat spelt xylan l⁻¹ (pH 6.5). The strain was maintained on minimal medium agar containing oat spelt xylan, as described previously (Flores *et al.*, 1997), at 4 °C and as a spore suspension in YEME supplemented with 40 % (v/v) glycerol (Hopwood *et al.*, 1985) at -20 °C. Strain CH-M-1035^T has been deposited in the DSMZ culture collection (Braunschweig, Germany) as strain DSM 41796^T, in the Industrial Culture Collection UNAM-48/WFCC (Mexico DF, Mexico) as strain BM-B-384^T and in the Northern Regional Research Center culture collection (Peoria, IL, USA) as strain NRRL B-24196^T.

Growth and sporulation of strain CH-M-1035^T were observed on standard media (Table 1); aerial spore-mass colour, pigmentation of substrate mycelium and the production of diffusible pigments were recorded following incubation of the strain at 37 °C for 7 days and, for some media, at 50 °C for 4 days. Peptone/yeast extract/iron agar and tyrosine agar (Shirling & Gottlieb, 1966) were used to score for the production of melanin pigments. Spore-chain morphology and spore-surface ornamentation were examined by scanning and transmission electron microscopy of 5-day-old cultures grown on minimal medium with 1 % (w/v) fructose. Samples were processed according to

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The GenBank accession number for the 16S rDNA sequence of *Streptomyces mexicanus* CH-M-1035^T is AF441168.

A fuller phylogenetic tree showing the placement of *Streptomyces mexicanus* CH-M-1035^T within the genus *Streptomyces* (over 100 species included) can be found in IJSEM Online (<http://ij.s.sgmjournals.org>).

Table 1. Growth and some phenotypic characteristics of strain CH-M-1035^T on different media

Agar medium	Growth*	Aerial hyphae	Substrate mycelium	Soluble pigments
YEME (ISP2)	+++	Grey	Yellowish-brown	Yellow
Oatmeal (ISP3)	+++	Grey	Yellowish-brown	Yellow
Starch (ISP4)	+/-	Not formed	Beige	None
Glycerol/asparagine	+	White/grey	Beige	None
Potato glucose	+++	Grey	Yellowish-brown	None
Modified Bennett's	+++	Grey	Yellowish-brown	Yellow
Tryptic soy	+/-	Not formed	Beige	None
Czapek-Dox/fructose	+	Grey	Beige	None
Corn meal	+	Grey	Beige	None
Nutrient	+/-	Not formed	Beige	None
Sabouraud	-		No growth	

* + + +, Good growth; +/-, variable growth; +, weak growth; -, no growth.

Kellenberger's technique (Nun, 1975). Whole-cell carbohydrates of the strain and the isomeric form of the diaminopimelic acid were determined according to Stanek & Roberts (1974).

Strain CH-M-1035^T was examined for a set of phenotypic properties, which are listed in Tables 1 and 2. The degradation tests were carried out at 37 °C as described by Williams *et al.* (1983). Growth on sole carbon sources was examined

on carbon utilization medium as recommended by Shirling & Gottlieb (1966). Antibiotic resistance was determined at 37 °C on modified Bennett's agar (MBA) by the disc method (Al-Tai *et al.*, 1999). The ability of strain CH-M-1035^T to inhibit the growth of three bacterial, one yeast, one yeast-like fungus and two fungal strains was detected by using the plug technique. The bacterial, yeast and fungal strains were inoculated onto Difco nutrient, Sabouraud glucose and potato glucose agar plates, respectively. Agar discs (8 mm)

Table 2. Phenotypic properties useful in distinguishing strain CH-M-1035^T from related *Streptomyces* spp.

Strains: 1, CH-M-1035^T; 2, *S. thermodiastaticus* DSM 40573^T; 3, *S. thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T; 4, *S. thermovulgaris* DSM 40444^T; 5, *S. thermocrophilus* DSM 41700^T; 6, *S. thermoalcalitolerans* DSM 41741^T. ND, Not determined; v, variable; -, negative; +, positive. Data for strain CH-M-1035^T are from this study; data for all other strains are from Kim, B. *et al.* (1999, 2000).

Property	1	2	3	4	5	6
Pigmentation of substrate mycelium	Beige	Yellow-brown	Yellow-purple	Not distinctive	Not distinctive	Not distinctive
Spore chain	<i>Rectiflexibiles</i>	<i>Spirales</i>	<i>Spirales</i>	<i>Spirales</i>	<i>Rectiflexibiles</i>	<i>Spirales</i>
Spore surface	Smooth	Tuberculate	Tuberculate	Smooth	Smooth	Warty
Melanin production	-	-	-	-	+	-
Growth on sole carbon source:						
L-Arabinose	+	-	-	-	+	+
Raffinose	+	+	-	-	-	-
L-Rhamnose	-	-	-	-	ND	+
Sucrose	-	-	-	+	-	+
Degradation of:						
Adenine	+	+	+	v	-	-
Guanine	-	-	-	-	ND	-
Hypoxanthine	+	-	-	-	+	-
Testosterone	-	+	-	v	-	+
Xylan	+	-	-	-	+	+
Growth at:						
20 °C	+	-	+	-	+	-
55 °C	+	+	+	+	-	+
pH 10	-	-	-	-	ND	+
7 % NaCl	-	-	-	-	+	ND

of a 7-day-old culture of strain CH-M-1035^T that had been grown on MBA at 37 °C were placed centrally onto the seeded cultures and the preparations were then incubated at 37 °C for 24 h (bacteria), 29 °C for 48 h (yeast) and 29 °C for 5 days (fungi). Growth of strain CH-M-1035^T at temperatures between 5 and 60 °C was determined on standard media (Shirling & Gottlieb, 1966). Tolerance of the novel strain to NaCl was determined on MBA at concentrations up to 13 %. The pH requirements of the strain were tested on sodium-phosphate-buffered MBA and on minimal agar medium containing 2.64 g (NH₄)₂SO₄ l⁻¹, 1.00 g MgSO₄·7H₂O l⁻¹ and 1.00 g KCl l⁻¹ supplemented with 1 % fructose (sterilized separately) and corresponding buffers (O'Donnell *et al.*, 1993). In the case of minimal medium, the buffering components were filter- or autoclave-sterilized separately to avoid chemical modifications and mixed into the cooled agar medium immediately before pouring. When necessary, K₂HPO₄ was added to the media to a final concentration of 0.07 % as a phosphate supplement.

Isolation of chromosomal DNA from strain CH-M-1035^T was carried out essentially as described by Hunter (1988). The 16S rRNA gene of strain CH-M-1035^T was amplified from purified DNA using the primers 5'-AGAGTT-TGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTAC-GACTT-3' (Weisburg *et al.*, 1991), and *Pwo* DNA polymerase (Roche Molecular Biochemicals). The PCR conditions used were: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1.5 min, annealing at 57 °C for 1.5 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. The amplified fragment was purified by gel electrophoresis and cloned into pMOSBlue (Amersham Pharmacia Biotech). Randomly picked individual clones were sequenced with universal primers T7 and -21, and with internal primers, using an ABI Prism BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The sequences were obtained with a model 310 Genetic Analyzer automated sequencer (Applied Biosystems).

The 16S rDNA sequence of strain CH-M-1035^T generated in this work (1450 nt; GenBank accession no. AF441168) was aligned with the 16S rDNA sequences of other streptomycetes retrieved from the EMBL/GenBank dataset using the PILEUP algorithm included in the GCG software package, version 7 (Devereux *et al.*, 1984); the resulting alignment was adjusted manually. The sequences corresponding to the primers used for amplification of the 16S rRNA gene were omitted from the alignment. The resulting alignment contained 1303 nt from 102 species. Phylogenetic analysis of strain CH-M-1035^T with its nearest neighbours was carried out using PAUP*, version 4.0b10 (Swofford, 2002). To determine which model of sequence evolution best fitted our dataset, a nested likelihood ratio test was performed using MODELTEST, version 3.04 (Posada & Crandall, 1998). Phylogenetic relationships were inferred using the maximum-likelihood approach (Felsenstein, 1981). Ten random taxon addition heuristic searches with

tree bisection-reconnection (TBR) branch swapping were conducted. The robustness of the inferred tree was evaluated using 1000 bootstrap replicates (Felsenstein, 1985).

The colonial morphology of strain CH-M-1035^T was consistent with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989). It formed a highly branched substrate mycelium and aerial hyphae which differentiated into long *Rectiflexibiles* chains carrying 10 or more rounded (mean diameter 0.88 µm), smooth-surfaced spores (Fig. 1). The spores were green upon examination under a light microscope. The colour of the substrate mycelium was generally beige or appeared yellow-brown when modified with soluble pigments, the aerial spore mass was grey-coloured with an appreciable greenish tint, and light yellow diffusible pigments were produced on several standard media (Table 1). Strain CH-M-1035^T contained LL-diaminopimelic acid but no characteristic sugars were found in the strain.

Fig. 1. Scanning (A) and transmission (B) electron micrographs showing *Rectiflexibiles* aerial mass with rounded, smooth spores of strain CH-M-1035^T. The organism was grown on minimal medium with 1 % fructose at 37 °C for 5 days.

To confirm that strain CH-M-1035^T was a streptomycete, we sequenced the almost-complete 16S rDNA from this micro-organism and compared it to the 16S rDNA sequences of previously described streptomycetes; on the basis of the 16S data, we showed that strain CH-M-1035^T was similar to other members of the genus *Streptomyces*. It was evident from the phylogenetic tree (Fig. 2) that strain CH-M-1035^T was closely related to the thermophilic streptomycetes that belong to the *Streptomyces thermodiastaticus* clade (Kim *et al.*, 1998). Strain CH-M-1035^T shares high percentage nucleotide sequence similarity values with *Streptomyces thermocoprophilus* B19^T (=DSM 41700^T; 98.6%), *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T (98.7%), *S. thermodiastaticus* JM 4840 (99.2%) and *S. thermoviolaceus* subsp. *apingens* DSM 41392^T (99.2%). These values correspond to 11–20 nt differences out of 1450 nt positions. Nucleotide similarity values within this range have been reported for several described species of *Streptomyces* (Kim *et al.*, 1999, 2000) which can be separated from each other on the basis of DNA relatedness data and phenotypic properties (Goodfellow *et al.*, 1987; Kim *et al.*, 1999, 2000). A fuller phylogenetic tree showing the placement of strain CH-M-1035^T within the genus *Streptomyces* (over 100 species included) can be found as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>).

Fig. 2. Relationship of *Streptomyces* sp. CH-M-1035^T with its nearest phylogenetic relatives in the single best tree resulting from maximum-likelihood analysis based on partial 16S rDNA sequences (1303 nt). The $-ln$ likelihood value was 2901.44. Branch lengths are proportional to the inferred amount of nucleotide substitution; numbers adjacent to branches indicate bootstrap values (expressed as a percentage of 1000 replicates). Bar, 0.005 substitutions per site.

Strain CH-M-1035^T had a number of phenotypic characteristics that could be used to distinguish it from related members of the *S. thermodiastaticus* clade (Table 2), on the basis of a numerical classification using the simple matching coefficient (Goodfellow *et al.*, 1987). The numerical analysis showed that strain CH-M-1035^T was phenotypically most closely related to *S. thermocoprophilus* B19^T, another xylanolytic organism (Kim *et al.*, 2000). However, strain CH-M-1035^T and *S. thermocoprophilus* B19^T could be separated on the basis of differences in melanin production, growth temperature, tolerance to NaCl, utilization of carbon sources and substrate degradation (Table 2). Moreover, separation of these two strains, as well as of strain CH-M-1035^T from other members of the clade, in the 16S-rDNA-based phylogenetic analysis was supported by significant bootstrap values (Fig. 2). The genotypic and phenotypic data suggest that strain CH-M-1035^T should be recognized as a novel species of the genus *Streptomyces*, for which we propose the name *Streptomyces mexicanus*.

Strain CH-M-1035^T grew well on simple minimal media containing ammonium sulphate, potassium phosphate and a wide range of carbohydrates; it is also important to mention that strain CH-M-1035^T grows well with xylan as sole carbon source (Flores *et al.*, 1997). The novel strain grew well up to 55 °C, although it did not form spores at elevated temperatures. Growth of strain CH-M-1035^T occurred in media with pH values between 4.3 and 8.0, and in the presence of 6% (w/v) NaCl. Strain CH-M-1035^T was resistant to ampicillin, but sensitive to erythromycin, kanamycin, gentamicin, thiostrepton, tetracycline, nalidixic acid, chloramphenicol and rifampicin. It did not produce antibiotics that were active against *Escherichia coli* JM 109, '*Sarcina lutea*' NRRL-B-1018, *Bacillus subtilis* ATCC 6633, *Aureobasidium* sp. CH-M-1018, *Aspergillus flavipes* ATCC 16795^T, *Trichoderma viridae* or *Pichia pastoris* GS 115.

Description of *Streptomyces mexicanus* sp. nov.

Streptomyces mexicanus (mex.i.ca'nus. N.L. adj. *mexicanus* belonging to Mexico, the source of the soil from which the organism was isolated).

Aerobic, Gram-positive, moderately thermophilic actinomycete. Forms highly branched substrate mycelium and aerial hyphae which differentiate into long *Rectiflexibiles* chains of 10 or more green, smooth spores. Spores are spherical, about 0.88 µm in diameter, or slightly oval. Aerial spore-mass colour is grey with a slight green tint. Substrate mycelium is beige on standard media. Yellowish diffusible pigments are formed on yeast-extract, malt-extract and oatmeal agar. Melanin pigments are not produced on peptone/iron or tyrosine agars. Positive for H₂S production. Degrades arbutin, starch, xylan, adenine, casein, hypoxanthine and L-tyrosine, but not guanine or testosterone. Utilizes L-arabinose, fructose, glucose, raffinose, mannitol, mannose, xylose, galactose, maltose, glycerol, lactose, cellobiose, trehalose and sodium acetate as sole carbon source, but not *meso*-inositol, melezitose, L-rhamnose,

sorbitol or sucrose. Growth occurs between 20 and 55 °C and from pH 4.3 to 8.0, and in the presence of 6 % (w/v) NaCl and ampicillin (100 µg ml⁻¹). Growth is inhibited in the presence of chloramphenicol, erythromycin, gentamicin, nalidixic acid sodium salt, kanamycin sulphate, rifampicin, tetracycline hydrochloride and thiostrepton, and in the presence of 7 % (w/v) NaCl. No antimicrobial activity is shown against *Escherichia coli* JM 109 or against representative strains of *Aspergillus flavipes*, *Aureobasidium* sp., *Bacillus subtilis*, *Pichia pastoris*, '*Sarcina lutea*' or *Trichoderma viridae*. Isolated from a soil sample obtained at a sugar-cane field in the State of Morelos, Mexico. The type strain is CH-M-1035^T (=DSM 41796^T =BM-B-384^T =NRRL B-24196^T).

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